

**Ischaemic preconditioning: An  
investigation of the patterns of kinase  
activation and protein expression profiles  
during reperfusion in the rat heart**

Susanna Maria Hattingh

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Promotors: Prof. Stefan du Plessis  
Prof. Anna-Mart Engelbrecht  
Dr. Ruduwaan Salie

Division of Medical Physiology  
Faculty of Medicine and Health Sciences  
Dept. of Biomedical Sciences  
University of Stellenbosch

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## **Declaration**

I, the undersigned, hereby declare that this study project is my own original work and that all sources have been accurately reported and acknowledged, and that this document has not been previously in its entirety or in part submitted at any university in order to obtain an academic qualification.

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## Abstract

**Introduction:** Coronary heart disease (CHD) is the leading cause of death worldwide with 3.8 million men and 3.4 million women dying globally each year. Although existing myocardial reperfusion strategies such as thrombolysis and percutaneous coronary intervention (PCI), if applied in a timely manner, limit myocardial infarct size, the mortality and morbidity remains significantly high. Ischaemic preconditioning (IPC) may offer the potential to attenuate myocardial ischaemia/reperfusion injury through cardioprotective signaling pathways which is recruited at the time of myocardial reperfusion, thereby improving clinical outcomes in patients with coronary artery disease.

Ischaemic preconditioning is a phenomenon whereby short intermittent episodes of coronary occlusion followed by reperfusion protect the myocardium against a subsequent period of sustained ischaemia. This protection is reflected in the limitation of infarct size and improved functional recovery of the ischaemic heart during reperfusion. Despite intensive research efforts, the promise of an effective cardioprotective strategy using the endogenous protective mechanisms of the heart which underlies IPC, has not yet been materialized. Although progress has been made in terms of signaling mechanisms in the preconditioned heart, the identification of the myocardial reperfusion phase as the critical “window” for cardioprotection, requires the elucidation of the signal transduction pathways during the reperfusion phase after IPC.

In view of the above, the aims of the present study were to investigate:

- i. the involvement of the RISK pathway and p38 MAP kinase pathway in IPC during early and late reperfusion
- ii. the involvement of heat shock protein-27 (HSP-27), heat shock protein-70 (HSP-70), GSK-3 $\beta$ , CAMKII, AMPK and the transcription factor CREB in the context of IPC during early reperfusion
- iii. the involvement of autophagy and apoptosis during early and late reperfusion after IPC

- iv. the correlation of the protein kinases with the hemodynamic parameters of the heart
- v. the mechanism of IPC by means of two-dimensional (2D) proteomics

**Methods:** The isolated perfused working rat heart model was used with functional recovery as end-point. Hearts were preconditioned (IPC) for 3x5 min global ischaemia, alternated with 5 min reperfusion. Hearts were subjected to 25 min sustained global ischaemia, followed by 5, 10, 15 or 30 min reperfusion when hearts were snap-frozen for western blotting analysis. Alternatively, hearts were reperfused for 30 min to record hemodynamic parameters and measure functional recovery. Non-preconditioned (Non-IPC) hearts were stabilized for 30 min and subjected to 25 min sustained global ischaemia followed by 5, 10, 15 or 30 min reperfusion when hearts were snap-frozen. Alternatively Non-IPC hearts were reperfused for 30 min to serve as control for the 30 min reperfused IPC group. Activation of the protein kinases was determined by western blotting analysis.

For the proteomic study mitochondrial and cytosolic proteins were isolated from heart tissue and separated in the first dimension by isoelectric focusing, followed by separation in the second dimension by two dimensional gel electrophoresis. The PD Quest software programme was used to identify significantly expressed protein spots. Protein spots of interest were excised and subjected to in-gel digestion and the resulting peptides were analysed by mass spectrometry. Proteins were identified by Mascot and the Swiss Prot database.

**Results:** Western blotting analysis demonstrated that the RISK pathway and p38 MAPK are activated very early in reperfusion, but the activation is not sustained during the reperfusion period. Autophagy is also upregulated during this early reperfusion phase; it is attenuated in the middle reperfusion phase and increase for a second peak of upregulation in the late reperfusion phase. In addition, we identified CAMKII as a novel marker of functional recovery in IPC after reperfusion.

The proteomic analysis identified twenty differentially expressed mitochondrial and thirty six differentially expressed cytosolic proteins between Non-IPC and

IPC hearts. Functions ascribed to the majority of these individual proteins were directly related to cardiac metabolism.

**Conclusion:** Activation of the majority of the protein kinases investigated in the present study is associated with the hemodynamic parameters of the heart instead of functional recovery. Results indicated that the variable signaling patterns could be attributed to differences in heart rate and the effect thereof (ejection fraction, minimum and maximum rate of contraction), as a result of sympathetic stimulation due to psychological stress in the animals before slaughtering. Proteomics results demonstrated that IPC hearts which failed after ischaemia /reperfusion are metabolically compromised and “worse off” compared to non-IPC hearts.

## Uittreksel

**Inleiding:** Koronêre hartsiekte is die vernaamste oorsaak van sterftes wêreldwyd met 3.8 miljoen mans en 3.4 miljoen vrouens wat jaarliks sterf. Alhoewel bestaande miokardiale herperfusie strategieë soos trombolise en perkutane koronêre intervensie (PKI), wanneer betyds toegepas, miokardiale infarkt grootte beperk, bly mortaliteit en morbiditeit steeds hoog. Isgemiese prekondisionering (IPK) beskik oor die potensiaal om miokardiale isgemie/herperfusie skade te verminder deur beskermende seinoordragpaaie tydens miokardiale herperfusie te aktiveer en sodoende die pasiënte wat aan koronêre arterie siekte ly, se prognose te verbeter.

Isgemiese prekondisionering verwys na die verskynsel waartydens kort episodes van isgemie opgevolg deur herperfusie, die miokardium teen 'n daaropvolgende langdurige isgemiese insident beskerm. Hierdie beskerming word gereflekteer in die beperking van infarkt grootte en verbeterde funksionele herstel van die isgemiese hart tydens herperfusie. Ten spyte van intensiewe navorsingspogings is die presiese meganisme van endogene beskerming tydens IPK nog nie ten volle ontrafel nie. Die identifisering van die miokardiale herperfusie fase se kritiese “vensterperiode” van beskerming, noodsaak 'n volledige analise van die seinoordragpaaie wat geaktiveer word tydens die herperfusie fase na IPK.

In die lig van bogenoemde, was die doel van die huidige studie om die volgende te ondersoek:

- i. die betrokkenheid van die RISK seinoordragpad en p38 MAP kinase tydens vroeë en laat herperfusie na IPK
- ii. die betrokkenheid van “heat shock protein-27” (HSP-27), “heat shock protein- 70” (HSP-70), GSK -3 $\beta$ , CAMKII, AMPK en die transkripsie faktor, CREB, in die konteks van IPK tydens vroeë herperfusie
- iii. die betrokkenheid van outofagie en apoptose tydens vroeë en laat herperfusie na IPK
- iv. die korrelasie van die proteïenkinases met die hemodinamiese parameters van die hart

- v. die meganisme van IPK deur middel van twee dimensionele proteomika

**Metodes:** Die geïsoleerde werkende rothart model, met funksionele herstel as eindpunt, is gebruik. Harte is geprekondisioneer (IPK) met 3x5 min globale isgemie, afgewissel met 5 min herperfusie. Daarna is harte blootgestel aan 25 min volgehoue globale isgemie, gevolg deur 5, 10, 15 of 30 min herperfusie, waartydens harte gevriesklamp is. Alternatiewelik, is harte blootgestel aan 30 min herperfusie ten einde funksionele herstel te meet en hemodinamiese parameters te registreer. Nie-geprekondisioneerde (Non-IPK) harte is gestabiliseer vir 30 min, waarna dit onderwerp is aan 25 min volgehoue globale isgemie, gevolg deur 5, 10, 15 of 30 min herperfusie, waartydens harte gevriesklamp is vir westelike klad analise. Alternatiewelik, is Non-IPK harte onderwerp aan 30 min herperfusie om te dien as kontrole vir die 30 min IPK groep. Aktivering van die proteïenkinases is bepaal deur westelike klad analise.

Vir die proteomiese studie, is onderskeidelik mitokondriale en sitosoliese proteïene geïsoleer en geskei in die eerste dimensie met behulp van isoelektriese fokusering, gevolg deur skeiding in die tweede dimensie met behulp van twee dimensionele gel elektroforese. Die PDQuest sagteware program is gebruik om proteïenkolle te identifiseer wat statisties beduidende verskille toon. Proteïenkolle van belang is uitgesny en onderwerp aan in-gel tripsinering en die peptiede wat sodoende verkry is, is deur middel van massa spektrometrie geanaliseer. Proteïene is geïdentifiseer deur Mascot en die Swiss Prot databasis.

**Resultate:** Westelike klad analise het aangetoon dat die RISK pad en p38 MAPK geaktiveer is tydens vroeë herperfusie, maar die aktivering word nie volgehou tydens die hele herperfusie periode nie. Outofagie word gestimuleer tydens die vroeë herperfusie fase; dit word onderdruk in die middel herperfusie fase en bereik 'n tweede piek van stimulering in die laat herperfusie fase. Die proteomiese analise het onderskeidelik twintig differensieel gereguleerde mitokondriale proteïene en ses en dertig differensieel gereguleerde sitosoliese proteïene geïdentifiseer tussen Non-IPK en IPK. Die grootste persentasie van hierdie proteïene is direk betrokke by miokardiale energie metabolisme. CAMKII is geïdentifiseer as 'n unieke merker van funksionele herstel in IPK tydens reperfusie.

**Gevolgtrekking:** Aktivering van die meeste van die proteïenkinases wat ondersoek is in die huidige studie, is geassosieer met die hemodinamiese parameters van die hart, in plaas van funksionele herstel. Die resultate het aangetoon dat die veranderende patrone van kinase aktivering toegeskryf kan word aan verskille in harttempo en die effek daarvan (ejeksie fraksie, minimum en maksimum tempo van kontraksie), as gevolg van simpatiese stimulasie toegeskryf aan sielkundige stres in die diere voor slagting. Proteomiese analise het getoon dat IPK harte wat faal na isemie/reperfusie metabolies gekompromiseer is en “slegter daaraan toe” is, in vergelyking met Non-IPK harte.



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**Figure 5.3.11c** Correlation of HSP-70 expression after 30 min retrograde perfusion with dP/dT min measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.11d** Correlation of HSP-70 expression after 30 min retrograde perfusion with heart rate measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.11e** Correlation of HSP-70 expression in the IPC group at 10 min reperfusion with heart rate measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.12** Correlation of CREB after 25 min sustained ischaemia with dP/dTMax measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.13** Correlation of GSK-3 $\beta$  in the IPC group at 30 min reperfusion with heart rate measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.14a** Correlation of AMPK after 30 min retrograde perfusion with dP/dTmax after baseline/stabilisation (15 min working heart).

**Figure 5.3.14b** Correlation of AMPK after 3xIPC with Aortic diastolic pressure measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.14c** Correlation of AMPK after 3xIPC with PSP measured at baseline/stabilisation (15 min working heart).

**Figure 5.3.15a** Correlation between CAMKII after 30 min reperfusion and aortic output at 30 min reperfusion.

**Figure 5.3.15b** Correlation between CAMKII activation after 30 min reperfusion and cardiac output at 30 min reperfusion.

**Figure 5.3.15c** Correlation between CAMKII activation after 30 min reperfusion and total work at 30 min reperfusion.

**Fig. 5.3.16** Comparison of heart rate and ejection fraction, measured at baseline/stabilisation (15 min working heart) between IPC hearts which showed functional recovery and failed respectively, after 30 min reperfusion.

**Figure 5.3.17** Correlation of heart rate with total work, measured at baseline/stabilisation (15 min working heart) between IPC hearts which showed functional recovery and failed respectively, after 30 min reperfusion.

## Abbreviation List

### Units of measurement:

%	percentage
$\mu\text{l}$	microliter
$\mu\text{g}$	microgram
ml	milliliter
g	gram
M	molar
Min	minute
hr	hour
mM	millimole
$\mu\text{M}$	micromole

### Chemical compounds:

$\text{Ca}^{2+}$	calcium
$\text{CO}_2$	calcium chloride
$\text{H}_2\text{O}$	water
$\text{K}^+$	Potassium
KCL	Potassium chloride

MgSO <sub>4</sub>	Magnesium sulphate
NaCl	Sodium chloride
NaHCO <sub>3</sub>	Sodium hydrogen carbonate
O <sub>2</sub>	Oxygen
Tris	tris(hydroxymethyl) aminomethane hydrochloride

**Other abbreviations:**

Non-IPC	Non preconditioning
IPC	Ischaemic preconditioning
SWOP	second window of protection
HF	heart failure
Ado	adenosine
ACs	adenylyl cyclases
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G

PI3-K	phosphoinositide 3-kinase
PKB/Akt	protein kinase B
MAPK	Mitogen-activated protein kinase
ERK	extracellular signal-regulated kinases
JNK	c-Jun amino-terminal protein kinase
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
NO	nitric oxide
ROS	Reactive Oxygen Species
HSP	heat shock protein
RISK	Reperfusion Induced Salvage Kinases
mito K <sub>ATP</sub> channels	mitochondrial K <sub>ATP</sub> channels

# **Introduction to the study and statement of the problem**

## **Introduction**

Ischaemic preconditioning (IPC) is a phenomenon whereby brief episodes of ischaemia and reperfusion protect the heart against a subsequent prolonged ischaemic insult. This protection is associated with a decrease in infarct size and improved functional recovery during reperfusion. Despite intensive research efforts the precise mechanism of IPC still remains elusive. It is proposed that events during the brief preconditioning ischaemic episodes, trigger signaling pathways which converge at the level of the mitochondria.

Although IPC is a powerful protective strategy, it could not be effectively employed in patients during acute myocardial infarction, since IPC has to be introduced before the onset of ischaemia and it is impossible to predict the time of infarction. Emerging studies suggest that events occurring during the post-ischaemic reperfusion phase, underlie the mechanism of IPC-induced protection.

## **Objectives and statement of the problem**

Research efforts to elucidate the mechanisms whereby IPC protects the heart have been hampered by the opposing results obtained by different research groups. This phenomenon has been attributed to differences in species (rabbits vs rats vs mice), models (Langendorff vs working heart mode), experimental protocols (single vs multi-cycle) and severity of ischaemia (global vs regional).

Of major concern are the conflicting data regarding kinase activation in the same species and model. Furthermore, researchers often report only one time point of kinase activation during reperfusion and then associate it with protection. We, therefore, hypothesize that kinase phosphorylation at reperfusion after IPC is a time dependent, dynamic process which correlates with different functional parameters.

## The aims of this study therefore were:

- 1) to characterize the activation patterns of the various **kinases** at different time points during reperfusion after IPC and to compare it with non-preconditioned hearts (Non-IPC) at the same time points during reperfusion after global ischaemia;
- 2) to assess **autophagy** at these different time points during reperfusion after IPC and to compare it with non-IPC hearts at the same time points during reperfusion after global ischaemia;
- 3) to assess **apoptosis** after 30 minutes of reperfusion after IPC and to compare it with non-IPC hearts at the same time point during reperfusion after global ischaemia;
- 4) to identify the **underlying mechanisms involved** contributing to poor functional recovery of the IPC hearts during early (15 min) reperfusion, by employing 2D proteomics;
- 5) to assess whether activation of the various **kinases** and **heat shock proteins** **correlate** with functional recovery and/or other haemodynamic parameters of the heart; i) before and after sustained ischaemia, ii) after the IPC protocol (3xIPC) and also iii) after IPC during early (5 min and 10 min ) and late (30 min) reperfusion;
- 6) to identify the **kinase(s)** of which the activation patterns do correlate with functional recovery of the ischaemic heart after IPC during 30 min reperfusion.

## Plan of study

As a background to the study a broad and thorough overview of the current literature on IPC and the signaling of IPC is provided in Chapter 1. Following this, the basic materials used and methods employed during the research project are outlined in Chapter 2. The experiments have been presented in three separate chapters. Each chapter has its own introduction, results and discussion section. Despite being separate chapters cognizance must be taken of the fact that the data is still closely related and supporting the universal goal of the study. The central theme of Chapter 3 was to assess kinase profiles through western blots while Chapter 4 examines the different protein expression profiles by means of proteomics between IPC and Non-IPC hearts. In chapter 5 the aim was to



correlate kinase activation with the haemodynamic parameters of the heart. The final conclusion and future directions were discussed in Chapter 6.

## **Conclusion**

Activation of the majority of the protein kinases investigated in the present study was associated with the hemodynamic parameters of the heart instead of functional recovery. Although the kinases play a major role in the protection elicited by IPC during the reperfusion phase, proteomic data also revealed an important role for proteins related to cardiac metabolism.

## **Ethical approval**

The study (project number: **P09/05/009**) has been approved by the Research Ethics Committee: Animal Care and Use (REC: ACU) of the University of Stellenbosch. Animals were handled according to the guidelines “for accepted standards for the use of animals in research and teaching”, as reflected in the South African National Standards (**SANS 10386:2008**) document.

# Chapter 1

## Introduction

Cardiovascular disease remains a leading cause of morbidity and mortality. Regardless of the latest advances in the prevention and management of heart disease, the World Health Organization predicts that it will be the major cause of death globally by the year 2020 [Murray et al., 1997], which will place an enormous economic burden on healthcare resources. As a result of this, there is continued interest in finding new drugs or developing novel interventions that will limit cardiac damage.

In the clinical setting, coronary reperfusion has proven to be the only means to reduce infarct size, after coronary artery occlusion. Reperfusion is a pre-requisite to salvage the ischaemic heart; however it is well established that reperfusion is accompanied by detrimental processes associated with reperfusion injury [Braunwald et al., 1985], which make the applications of cardioprotective strategies possible at this time. Consequently, numerous studies have investigated the potential of cardioprotective drugs or interventions administered at the onset of reperfusion to limit infarct size, such as thrombolysis or primary angioplasty.

Thrombolysis as reperfusion strategy in acute myocardial infarction involves the intravenous application of streptokinase or tissue plasminogen activator [Hartzler, 1983]. Direct primary percutaneous intervention, also known as coronary angioplasty, is the primary reperfusion therapy for acute myocardial infarction [Meyer et al., 1982]. Other interventions which can modify reperfusion injury include administration of  $\text{Na}^+\text{-H}^+$  exchange inhibitors [Karmazyn, 1988], transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) [Lefer et al., 1993], fibroblast growth factor-2 (FGF-2) [Cuevas et al., 1999], cardiotrophin-1 (CT-1) [Cai et al., 1999], Insulin growth factor-1 [Buerke et al., 1995], activation of kinases [Hausenloy et al., 2004], perfusion with erythropoietin [Hanlon et al., 2005]. These have been reported to protect the myocardium when administered during reperfusion. However, results from failed clinical trials [Bolli et al., 2004; Flaherty et al., 1994]

indicated that the window of opportunity during reperfusion is very limited. Therefore candidate treatments or agents would need to be very effective when administered during reperfusion.

Injury to the myocardium also occurs during ischaemia of which the proportion depends on the duration of the ischaemic event. In vivo studies with rabbits demonstrated that infusion of a glucose/insulin/potassium (GIK) cocktail 30 min before ischaemia continuing throughout the reperfusion phase elicited protection against ischaemia/reperfusion injury [Zhang et al., 2004]. A study by Jonassen and coworkers 2009, demonstrated that pre-treatment with insulin before ischaemia reduces infarct size in langendorff-perfused rat hearts.

Results from a pilot clinical study indicated that administration of GIK reduced mortality in patients with acute myocardial infarction undergoing reperfusion [Diaz et al., 1998]. This clinical study was supported by experimental in vivo studies with rats, which demonstrated that GIK infusion at reperfusion reduces infarct size [Jonassen et al., 2000]. A later study by the same group, demonstrated that infusion of insulin at the onset of reperfusion had the same protective effects of GIK treatment and reduced infarct size in langendorff-perfused rat hearts [Jonassen et al., 2001].

Even though several attempts [Hartzler, 1983; Meyer et al., 1982; Karmazyn, 1988; Lefer et al., 1993; Cuevas et al., 1999; Cai et al., 1999; Buerke et al., 1995; Hausenloy et al., 2004; Hanlon et al., 2005] to protect the ischaemic heart from lethal reperfusion damage have been intensively studied, the results were mostly controversial and unsatisfactory. Only a few of these results have been reproducible and none has been translated into clinical therapies [Bolli et al., 2004]. However, in 1986 it was discovered that the heart has an endogenous protective mechanism, known as ischaemic preconditioning or IPC [Murray et al., 1986].

## 1.) The phenomenon of ischaemic preconditioning (IPC)

Ischaemic preconditioning (IPC) is a phenomenon in which brief episodes of ischaemia and reperfusion protects the heart against a subsequent prolonged ischaemic insult [Murry et al., 1986]. This protection is reflected as a decrease in infarct size and an increase in functional recovery of the ischaemic heart after reperfusion [Shiki & Hearse, 1987]. Ischaemic preconditioning has been acknowledged as the “strongest” form of *in vivo* protection against myocardial ischaemic injury, apart from early reperfusion [Kloner et al., 1998]. This phenomenon has evoked much attention and has been the object of major scientific investigations, aiming at elucidating the mechanism of IPC, which could lead to the development of pharmacological interventions to be used in a clinical setting. IPC has been observed in a variety of organs including the bladder, the brain, the gut, the kidney, the liver, the retina, skeletal muscle and the skin [Hausenloy et al., 2008; Yellon & Downey, 2003]. IPC was shown to occur in all species tested including canine [Przyklenk et al., 1995], mice [Miller & Winkle, 1999], pigs [Valhaus et al., 1996], rabbit [Liu et al., 1991] and rat [Liu & Downey, 1992]. It was also demonstrated that functional recovery in isolated human atrial trabeculae was greatly enhanced after an extended period of hypoxia by earlier hypoxic preconditioning [Speechly-Dick et al., 1995]. Despite the ability to demonstrate the occurrence of IPC in different animal models, the translation to the human clinical setting has been largely disappointing [Andrew et al., 2010].

IPC occurs in two phases. The first phase, or classical preconditioning, is also known as the “early phase” or the “first window” of protection. The first phase is short-lived and cardioprotection wanes after 1-2 hours [Murray et al., 1986]. The second (late) phase or “delayed preconditioning”, also termed the “second window of protection” (SWOP), appears 12-24 hours after the IPC protocol and lasts for 48-72 hours [Marber et al., 1993; Yang et al., 2010].

Although, IPC is a powerful protective strategy, it could not be effectively employed in patients during acute myocardial infarction, since IPC has to be introduced before the onset of ischaemia. It is impossible to predict the time of infarction, but if IPC exerts its protective effect at reperfusion, then clinical

therapeutic salvage could still be possible by implementing protective strategies at reperfusion.

The signal transduction pathways of IPC can be divided into triggers, mediators and end effectors of protection. It has become an important objective to identify the triggers, mediators and end effector of IPC. **Triggers** are activated during the ischaemia/reperfusion cycle of the preconditioning phase, before the onset of sustained ischaemia. Inhibition of the triggers' action during the preconditioning phase will block or abolish its cardioprotective effect. **Mediators** are important during the period of sustained index ischaemia and during the first few minutes of reperfusion after sustained ischaemia. Similarly, inhibition of a mediators' action during this time will abolish the cardioprotection afforded by IPC. Elucidation of the signaling mechanisms involved in the cardioprotective effect of triggers/mediators in IPC can result in the development of pharmacological mimetics to be used in clinical therapy.

### **1.1) Signaling pathways involved in ischaemic preconditioning (Fig.1.1)**

It is generally believed that simultaneous stimulation of the adenosine-, bradykinin- and opioid receptors in combination with the release of reactive oxygen species (ROS) during the short episodes of ischaemia/reperfusion all contribute to the triggering of IPC. It was hypothesized that the resulting signaling cascade requires convergence of all stimuli on a common distal pathway. This pathway is thought to involve protein kinase C (PKC), since inhibition of PKC inhibit the cardioprotective effects of ROS [Baines et al., 1997], adenosine [Sakamoto et al., 1995], the opioid receptors [Miki et al., 1998] as well as bradykinin [Goto et al., 1995]. This hypothesis was supported by studies in the rat [Mitchell et al., 1995] and in the rabbit [Ytrehus et al., 1994] which concluded that PKC activation plays a crucial role in IPC-induced protection.

Adenosine, bradykinin and the opioids act via their corresponding G-protein coupled receptors to activate very divergent signaling cascades, which converges on a final common pathway. It is generally believed that adenosine receptors can activate PKC via phospholipases which produce diacylglycerol from membrane

phospholipids [Cohen et al., 2001]. Opioid receptors trigger through PI3-K activation and are dependent on transactivation of the epidermal growth factor receptor (EGFR) by metalloproteinases [Cohen et al., 2007]. Bradykinin is also dependent on PI3-K activation for its triggering action, but it occurs through an EGFR independent mechanism [Cohen et al., 2007]. The steps downstream in the signaling pathway through PI3-K appear to be the same for both bradykinin and the opioids, with subsequent phosphorylation of PKB/Akt through phospholipid dependent kinases. Activation of PKB/Akt results in the activation of eNOS, leading to the production of NO. NO stimulates guanylyl cyclase to produce cGMP, which in turn activates PKG [Cohen et al., 2001; Oldenburg et al., 2004].

Several other substances were also found to trigger IPC through PKC activation, including angiotensin II [Liu et al., 1995], the catecholamines [Banerjee et al., 1993] and endothelin [Wang et al., 1996]. However, these substances are not released in sufficient quantities during ischaemia to play a major role in IPC.

It is well documented that reactive oxygen species (ROS), previously categorized as a receptor independent trigger, can stimulate IPC-induced protection by transient exposure of the heart to ROS generating systems [Yang et al., 2012]. Several studies demonstrated that cardioprotection induced by IPC could be blocked by ROS scavengers [Tritto et al., 1997; Baines et al., 1997]. In addition, cardioprotection induced by ROS could be blocked by inhibition of PKC, indicating that ROS signaling occurred upstream of PKC [Kuno et al., 2008].

The source of ROS is thought to be the mitochondria. The mitochondrial  $K_{ATP}$  channel (mito  $K_{ATP}$ ) is located in the mitochondrial inner membrane and has been shown to play an important role in IPC-induced cardioprotection [Liu et al., 1998; Garlid et al., 1997]. Opening of the mito  $K_{ATP}$  channel results in influx of  $K^+$  which is balanced by electrogenic  $H^+$  efflux driven by the respiratory chain, resulting in the increased production of ROS [Pain et al., 2000; Forbes et al., 2001]. ROS has been shown to induce the activation of PKC- $\epsilon$  [Yue et al., 2002; Schultz et al., 2003]. It has been proposed that activation of PKC is associated with the last step of the trigger phase as well as with the first step of the mediator phase [Downey et al., 2008].

The target of PKC however, remains unclear. It has been shown that PKC-induced protection could be blocked by an adenosine receptor inhibitor [Philipp et al., 2006]. Since PKC inhibition does not affect protection mediated by the adenosine A<sub>2B</sub> receptor [Kuno et al., 2007], it is believed that the A<sub>2B</sub> receptor is located downstream of PKC. It is believed that PKC sensitizes the adenosine receptor to the heart's endogenous adenosine. PKC thus plays a major role in IPC and can also directly or indirectly modulate several components which are associated with mitochondrial membranes. These include the mito K<sub>ATP</sub> channel, BAX, BAD, Bcl-2 and also the mitochondrial permeability transition pore (MPTP) [Murphy 2004; Costa et al., 2005 & 2006], which are important modulators of cell survival/death.

It is generally believed that IPC protects the heart by activating the ERK p44/p42 MAPK- and PI3-K/PKB/Akt signaling pathways at reperfusion [Hausenloy et al., 2005]. These pathways combined are denoted as the "Reperfusion Injury Salvage Kinase" or RISK pathway. Inhibition of either of these pathways during early reperfusion abolishes the protection afforded by IPC. Therefore, it is proposed that IPC exerts its protection during early reperfusion, after the period of lethal ischaemia [Hausenloy et al., 2005]. This finding provided enormous hope for implementing IPC in the clinical setting, particularly when blood supply to the ischemic zone is restored after clinical procedures. In fact, since 2005 many pharmacological agents were tested and found to protect the myocardium when given during early reperfusion. These include insulin [Baines et al., 1999], the adenosine A<sub>1</sub>/A<sub>2</sub> agonist, Bay 60-6583 [Xu et al., 2000], the adenosine agonist 5'-(N-ethylcarboxyamido) adenosine (NECA) [Yang et al., 2004], bradykinin [Yang et al., 2004], cyclosporine A [Hausenloy et al., 2009], erythropoietin [Cai & Semenza, 2004], natriuretic peptide [Yang et al., 2006], transforming growth factor- $\beta$ 1 [Baxter et al., 2001] and urocortin [Schulman et al., 2002]. All of these agents, except cyclosporine A, depend on the activation of the RISK pathway to induce cardioprotection.

The mitochondrial permeability transition pore (MPTP) is currently postulated to play a crucial role in determining cell death or survival [Vigneron et al., 2011]. It was found that the MPTP remained closed during ischaemia and open only during the first few minutes of reperfusion. It has been shown that activation of

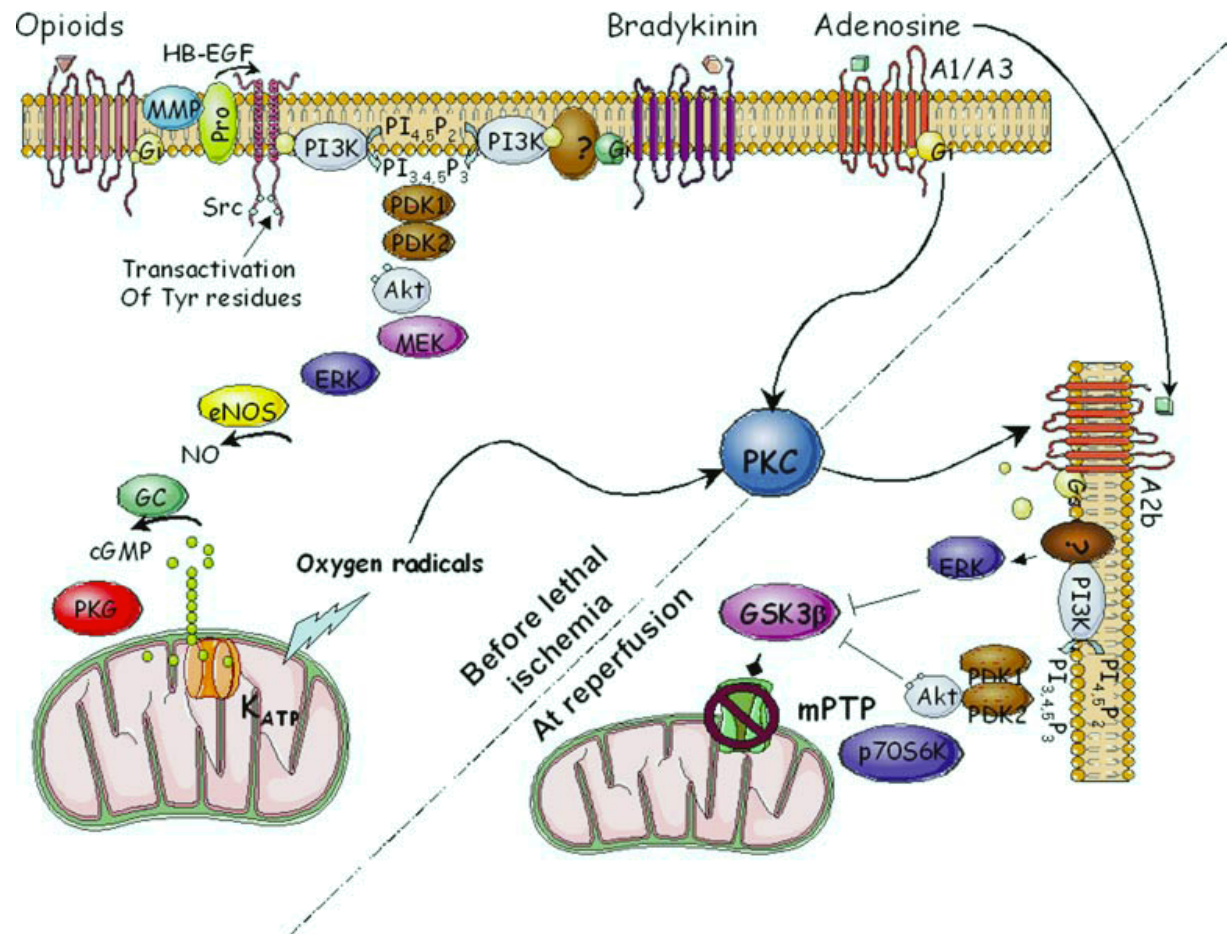
the RISK pathway and other protective signals converge to phosphorylate glycogen synthase kinase-3 $\beta$  on Ser<sup>9</sup>, resulting in its inactivation and subsequent inhibition of MPTP opening which results in cardioprotection [Juhaszova et al., 2004]. The immunosuppressant drug, cyclosporine A, also inhibited MPTP opening induced by oxidative stress, phosphatases and calcium [Crompton et al., 1988].

In summary, more than two decades after the description of ischaemic preconditioning (IPC) and despite all the intensive research efforts and knowledge gained, the exact mechanism of this endogenous protective phenomenon still remains to be fully elucidated.

According to Downey and co-workers (2008), the events during the triggering phase of IPC are well mapped. Events during the reperfusion phase are less well defined. Therefore, the finding that IPC exerts its protection during early reperfusion by activation of the RISK pathway caused a paradigm shift and has led to increased focus on events during early reperfusion.

A recurring theme throughout this literature review is the great controversy regarding the role of protein kinases as potential mediators of protection in the setting of IPC-induced protection. Many of the studies investigating the role of these kinases in IPC, followed a pharmacological approach. This has led to a variety of suggested receptors and signaling pathways that could possibly be involved in the mechanism of IPC. However, it is also believed that meticulous investigation of the events during an IPC protocol will identify the parameters crucial for cardioprotection.





**Figure 1.1:** A cartoon showing the sequence of signaling events involved in triggering the preconditioning state prior to the ischaemic insult (events above the dividing line) and those that mediate protection in the first minutes of reperfusion (events below the dividing line). See text for details. [Downey et al., 2008].

## **1.2.) Receptor dependent triggers of early preconditioning**

### **1.2.1.) $\alpha$ - and $\beta$ - Adrenoreceptors**

Adrenergic receptors belong to the superfamily of G protein-coupled receptors (GPCR). They share the common feature of 7-transmembrane spanning domains and are involved in physiological responses to autocrine and neurotransmitters [Yarden et al., 1986]

The  $\beta$ -adrenergic receptor ( $\beta$ -AR) is a typical G protein-coupled receptor (GPCR). Upon stimulation, via circulating catecholamines (sympathetic stimulation), it regulates a wide range of biological processes. These include myocardial contractility and relaxation, vascular and bronchial smooth muscle tone, pacemaker activity, cell growth, cell survival and cell death. It is also involved in metabolic regulation, such as glucose and lipid metabolism. Adrenergic receptors can be divided into  $\alpha$ - and  $\beta$ - adrenergic receptors [Ahlquist, 1948]. By using the appropriate agonists and antagonists the adrenergic receptors were further classified into  $\alpha$ 1-,  $\alpha$ 2-,  $\beta$ 1- and  $\beta$ 2- adrenergic receptor subtypes [Lands et al., 1967; Ablad et al., 1974]. The  $\beta$ 3 receptor was identified at a later stage [Emorine et al., 1989].

All three  $\beta$ -AR subtypes are found in a variety of tissue. They form a central part of membrane proteins located in the hearts of different species [Bylund et al., 1998]. Sympathetic stimulation of the  $\beta$ -adrenergic receptor by catecholamines (norepinephrine and epinephrine) functions as a powerful regulatory system to enhance cardiac output as a consequence of stress, injury or exercise [Hata et al., 2004; Lohse et al., 2003]. Sympathetic stimulation increases the release of norepinephrine and epinephrine from the adrenal medulla to all areas of the heart, whereas the parasympathetic nervous system normally opposes its effects to maintain blood pressure within the normal range. The parasympathetic nervous system acts via the vagal nerve to release acetylcholine to control blood pressure [Opie, 1998].

A G-protein coupled receptor (GPCR) is characterized by a glycosylated amino (N) terminus and an intracellular carboxyl (C) terminus region with serine and

threonine residues which are potential phosphorylation sites. The seven transmembrane domains (TD) are linked by three extracellular loops and three intracellular loops [Yarden et al., 1986]. Ligand binding triggers a conformational change in the GPCR with subsequent disruption of ionic interactions between the third cytoplasmic loop and the sixth transmembrane segment. This allows coupling with the heterotrimeric guanine-nucleotide regulatory proteins (G-proteins) [Han et al., 1998; Wess, 1997]. The phosphorylation sites of protein kinase A (PKA) is situated on the third intracellular loop of the receptor. It is therefore thought that PKA plays an important role in agonist induced uncoupling, subsequent rapid desensitization and downregulation of the receptor [Benovic et al., 1985].

The  $\beta_1$ -AR and  $\beta_2$ -AR coexist in cardiomyocytes of many mammalian species [Xiao et al., 1999], including in the human heart where the  $\beta_1$ -AR is the predominant receptor. It is equally distributed in all parts of the heart [Brodde, 1991]. The human heart expresses  $\beta_1$ -AR and  $\beta_2$ -AR at a ratio of about 70-80%:30-20% in the ventricles and 60-70%:40-30% in atria [Brodde, 1991]. The  $\beta_1$ -AR stimulates cardiac muscle and contributes to the relaxation of blood vessels, whereas the  $\beta_2$ -AR relaxes smooth muscle and contributes to cardiac contractility [Lands et al., 1967; Brodde, 1991].

The  $\beta_3$ -AR subtype is expressed in a variety of tissues and is found to a large extent in the coronary vascular bed [Strosberg, 1997]. The  $\beta_3$ -AR subtype stimulates the L-type calcium current in human atrial myocytes [Skeberdis et al., 1999] and reduces contractile force in human ventricular muscle [Gauthier et al., 1989].  $\beta_3$ -adrenergic receptors are also expressed in adipose tissue [Emorine et al., 1989]. It also mediates lipolysis in white adipose tissue and thermogenesis in brown adipose tissue [Arch, 1989].

Signaling by cardiac  $\beta$ -adrenergic receptors has been studied in great detail and it is generally accepted that the  $\beta$ -AR subtypes have different affinities for their ligands, resulting in the activation of distinct signaling pathways [Lohse et al., 2003]. It is proposed that spatial segregation of receptors allows their involvement with other segregated proteins, resulting in the formation of signalosomes [Lohse et al., 2003].

$\beta$ -AR signaling is initiated by binding of the agonists (catecholamines) to the receptor, followed by a subsequent conformational change, resulting in the activation of the classical Gs-adenylyl cyclase (AC)-cAMP-PKA signaling pathway. PKA, which is the primary target of cAMP, phosphorylates several proteins that are essential for cardiac function, including L-type calcium channels [Zhao et al., 1994], phospholamban [Simmerman et al., 1998], ryanodine receptors [Marx et al., 2000], troponin 1 [Sulakhe et al., 1995] and myosin binding proteins [Kunst et al., 2000]. This affects cardiomyocyte contractility by increasing the  $\text{Ca}^{2+}$  influx through the L-type channel and increasing  $\text{Ca}^{2+}$  re-uptake into the sarcoplasmic reticulum (phospholamban/SERCA) and modulating myofilament  $\text{Ca}^{2+}$  sensitivity (troponin I, myosin binding protein-C) [Lohse et al., 2003].

It is also well known that catecholamines act through  $\alpha$ -adrenergic receptors ( $\alpha$ -AR). Two classes of  $\alpha$ -AR have been identified,  $\alpha_1$ -AR and  $\alpha_2$ -AR [Shannon & Chaudhry, 2006]. The  $\alpha_1$ -AR can be further classified into three subtypes;  $\alpha_1$ -A,  $\alpha_1$ -B and  $\alpha_1$ -D with distinct physiological roles. The  $\alpha_1$ -AR plays an adaptive role in the heart and has been shown to protect against the development of heart failure in contrast with the detrimental effects of chronic  $\beta$ -AR stimulation. The beneficial effects of the myocardial  $\alpha_1$ -AR subtype have been confirmed by studies using knockout (KO) mice models [Simpson, 2006]. These studies demonstrated that KO of the two main myocardial  $\alpha_1$ -AR subtypes,  $\alpha_1$ -A and  $\alpha_1$ -B, resulted in the impairment of normal post-natal myocardial growth. Consequently, this resulted in severe dilated cardiomyopathy and death after pressure overload [O'Connell et al., 2003 & 2006]. This data obtained from KO studies suggested that the  $\alpha_1$ -AR is cardioprotective [Huang et al., 2007; O'Connell et al., 2006], whereas the role of the  $\alpha_1$ -AR appears to be associated with physiological cardiac hypertrophy [Vecchione et al., 2002; Rodrigo et al., 2005]. All three  $\alpha$ -AR subtypes can cause constriction of peripheral arteries [Vecchione et al., 2002], whereas the cardiac  $\alpha_1$ -AR stimulates coronary vasoconstriction [Turnbull et al., 2003]. Studies demonstrated that  $\alpha_1$ -A,  $\alpha_1$ -B and  $\alpha_1$ -D are all present in the human heart [Jensen et al., 2009]. In addition, the  $\alpha_1$ -AR subtype expression was shown to be similar to the mouse heart with  $\alpha_1$ -A and  $\alpha_1$ -B in the myocardium and the  $\alpha_1$ -D subtype in the coronary arteries. The  $\alpha_1$ -AR mRNA's are not repressed in heart failure and  $\alpha_1$ -AR are not downregulated in contrast with  $\beta$ -AR subtypes [Jensen et al., 2009].

A large percentage of studies proposed that activation of the  $\alpha$ - [Hu et al., 1995; Tsuchida et al., 1994] and  $\beta$ - adrenoreceptors [Spear et al., 2007; Tong et al., 2005] can act as triggers of IPC. This was based on the findings that IPC-induced cardioprotection was attenuated when these receptors were blocked. Other contradictory reports showed that neither depletion of intramyocardial catecholamine storage and release [Ardell et al., 1996] nor specific catecholamine receptor antagonists [Thornton et al., 1993] could block the protection afforded by IPC. It was, however, shown that exogenous catecholamines or adrenoreceptor agonists could precondition the heart where direct involvement of the  $\alpha$ 1-A and  $\alpha$ 1-B-receptors during IPC was suggested [Thornton et al., 1993 & Tsuchida et al., 1994]. Another study however, suggested the involvement of the  $\alpha$ 1-A-, rather than the  $\alpha$ 1-B receptor [Rorabaugh et al., 2005].

Activation of the  $\beta$ 2-adrenoreceptor was also reported to be important in IPC-induced cardioprotection [Tong et al., 2005]. B1-adrenoreceptor activation also conferred protection during the preconditioning period, but activation was shown to be deleterious after reperfusion [Spear et al., 2007].

Although the  $\alpha$ 1-adrenergic receptor was proposed to play a role in IPC-induced cardioprotection, our laboratory could not find evidence for this receptor in the mechanism of IPC [Moolman et al., 1996].

In addition, the steps during an IPC protocol were meticulously investigated in our laboratory to serve as a guide to direct future research [Moolman et al., 1996]. The finding that cAMP increased in a cyclic manner at the end of each IPC episode suggested a potential role for  $\beta$ -adrenergic receptor signaling as trigger in the IPC process [Lochner et al., 1998 & 2000]. It was first reported by Asimakidis et al. [1994] that pharmacological preconditioning with isoproterenol induced protection against ischaemia. Subsequently it was reported that transient  $\beta$ -AR stimulation with agents such as dobutamine and isoproterenol could mimic IPC and offer protection against a subsequent period of sustained ischaemia [Miyawaki & Ashraf, 1997; Lochner et al., 1999]. This phenomenon is known as  $\beta$ -preconditioning.

### **1.2.2.) Adenosine, Bradykinin and the opioids**

Adenosine is an ubiquitous nucleoside which is continuously produced intracellularly and extracellularly under normal conditions. It is maintained at low intracellular levels (100-300 nM) by the enzymes adenosine kinase and adenosine deaminase. Adenosine is released by metabolically active and stressed cells (oxygen deprivation) in which case the levels can increase up to 10  $\mu$ M [Zetterstrom et al., 1982; Hagberg et al., 1987]. Adenosine plays a major role in the cardiovascular system [Baines et al., 1999], the central nervous system [Fredholm et al., 1995], the gastrointestinal tract [Linden, 1994; Marquardt, 1998], the immune system [Cronstein, 1994], cell growth, proliferation and apoptosis [Burnstock, 2002] under normal and stressful situations.

In the heart, adenosine has the following electrophysiological effects; transient reversible slowing of heart rate (negative chronotropic effects), anti-arrhythmic effects and impairment of atrioventricular conduction (negative chronotropism) [Drury & Scent-Gyorgi, 1929; Belardinelli et al., 1989]. Adenosine mediates two types of actions in the heart, those that are cAMP dependent (direct effects) and also those that are cAMP independent (indirect effects).

In addition, adenosine has an essential function in the regulation of the myocardial oxygen supply, known as demand balance. This is achieved by increasing the oxygen supply through coronary vasodilation and reducing the oxygen demand by decreasing myocardial contractility. Adenosine also attenuates the effects of the catecholamines and thereby suppresses conduction inside the sinoatrial (SA) and atrioventricular (AV) nodes [Belardinelli et al., 1989]. Adenosine also inhibits oxygen metabolite production by activated neutrophils [Pelleg, 1985; Belardinelli et al., 1989], attenuates the release of norepinephrine from nerve terminals and activates glycolysis [Pelleg, 1985; Belardinelli et al., 1989]. The cardiac actions of adenosine were shown to be surprisingly similar to that of acetylcholine (Ach) [Belardinelli & Isenberg, 1983]. Therefore, it appears as if Ach and adenosine act in parallel to antagonize the effects of cardiac sympathetic stimulation.



Adenosine mediates its effect via activation of cell surface receptors namely A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> adenosine (AdoRs) receptors. These receptors belong to the G-protein-coupled superfamily of receptors [Auchampach & Bolli, 1999; Olah & Stiles, 1995].

The A<sub>1</sub>-AdoR is the most extensively studied adenosine receptor in the setting of cardioprotection. Several studies reported the involvement of the A<sub>1</sub>-AdoR with signaling cascades such as the DAG/IP<sub>3</sub>/PLC cascade and PKC, MAPKs, PI3-kinase and PKB/Akt [Buck, 2004; Mubagwa & Flemming, 2001; Headrick et al., 2003]. Activation of the mito K<sub>ATP</sub> channel and PKC also play a role in A<sub>1</sub>-AdoR mediated cardioprotection [Peart & Headrick, 2003].

Liu and co-workers (1991) discovered that activation of the Gi-coupled adenosine A<sub>1</sub> receptor triggered IPC's protection [Liu et al., 1991]. It has thus become clear that IPC in the heart was receptor mediated and the result of signal transduction pathways.

It was also reported that the A<sub>2A</sub>-AdoR protected against ischaemia/reperfusion injury by limiting the damaging effects of neutrophil activation [Lasley et al., 2001]. These receptors have also been shown to play a role in the cardioprotection afforded by postconditioning [Kin et al., 2005]. Activation of the A<sub>2A</sub>-AdoR has been shown to limit infarct size during reperfusion in the isolated rabbit heart and this cardioprotection was linked to activation of ERK p44/42 [Kis et al., 2003].

The A<sub>2B</sub>-AdoRs are less well studied in the setting of myocardial ischaemia/reperfusion [Kilpatrick et al., 2002]. However, the A<sub>2B</sub>-Ado has been shown to play a role in the infarct limiting effects of postconditioning in the rabbit heart. This protection implicated the involvement of PKC [Phillip et al., 2006]. The cardioprotective role of the A<sub>2B</sub>-Ado was also demonstrated in IPC induced protection during reperfusion [Solenkova et al., 2005].

Overexpression of the A<sub>3</sub>-AdoR enhances ischaemic tolerance and significantly limits ATP depletion during ischaemia [Gross et al., 2002]. This is consistent with adenosine dependent increase of myocardial energy state [Fralix et al., 1993].

The A<sub>3</sub>-AdoR was shown to be involved in delayed preconditioning in a similar fashion to the A<sub>1</sub>-AdoR [Takano et al., 2001].

Studies followed that indicated involvement of bradykinin B<sub>2</sub> [Wall, et al., 1994] and opioid- $\delta$ -receptors [Schulz et al., 1995] in IPC-induced protection. These substances can act as triggers of IPC by binding to their corresponding G-protein coupled receptors. This lead to the activation of signaling pathways that is thought to converge on a common target, possibly protein kinase C (PKC), since inhibition of PKC attenuated the protection afforded by IPC [Yang et al., 2010].

It was also shown that adenosine [Sakamoto et al., 1995], bradykinin [Goto et al., 1995] and the opioids [Miki et al., 1998] are released in sufficient quantities by ischaemia to act as endogenous triggers of IPC. Opioid receptor signaling is suggested to depend on transactivation of the epidermal growth factor receptor (EGFR), which activates PI3-kinase [Cohen et al., 2007]. This process is metalloproteinase-mediated and the EGFR tyrosine kinase auto-phosphorylates its tyrosine residues when bound to its triggering growth factor. Bradykinin's triggering action is also dependent on PI3-kinase activation, but it appears to be independent of EGFR. For both the opioids, as well as bradykinin, the steps downstream of PI3-kinase appear to be similar. PI3-kinase causes phosphorylation of PKB/Akt, which in turn activates endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO). NO stimulates guanylyl cyclase (GC) to produce cyclic guanosine monophosphate (cGMP), which stimulates protein kinase G (PKG) [Oldenburg et al., 2004].

There is evidence that cyclooxygenases may act as triggers of IPC in rats [Chen et al., 1999] and mice [Gabel et al., 2001]. Norepinephrine, angiotensin, prostaglandins and endothelin are also released during the brief periods of ischaemia/reperfusion during IPC. Endogenous prostaglandins however, do not act as triggers of IPC in the rat [Li et. al., 1992] or rabbit [Liu et al., 1992]. Agonists such as norepinephrine, endothelin and angiotensin can precondition the heart when administered exogenously, but are not released in sufficient quantities during ischaemia to act as endogenous triggers of IPC [Tanno et al., 2000; Wang et al., 1996; Moolman et al., 1996].



### **1.3.) Receptor independent triggers:**

#### **1.3.1.) Reactive oxygen species (ROS)**

Biological systems are extremely capable to tolerate the production of ROS under normal conditions and to maintain this well balanced system. However, during ischaemia/reperfusion this balance becomes disturbed as cellular antioxidant defense mechanisms are attenuated during ischaemia. The ability of elevated hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) to produce the destructive hydroxyl radical ( $\cdot\text{OH}$ ) becomes more favorable under these conditions [Hess & Manson, 1984].

ROS are extremely reactive molecules and include superoxide anion ( $\text{O}_2^{\cdot-}$ ) and the hydroxyl radical ( $\text{OH}\cdot$ ). However,  $\text{H}_2\text{O}_2$ , which is not considered a free radical, is often discussed in combination with ROS, since it is also very active. Free radicals originating from the reaction of oxygen with nitrogen are considered a subclass of free radicals and are known as reactive nitrogen species (RNS). RNS include nitric oxide (NO) and peroxynitrite ( $\text{ONOO}^-$ ). ROS and RNS are the products of several enzymes and biochemical processes. Biological systems have several defense mechanisms against reactive oxygen species under normal conditions. These protective mechanisms, known as anti-oxidants may be enzymatic or non-enzymatic. Anti-oxidants could also function as triggers of signaling cascades in the cell [Marczin et al., 2003].

The main source of ROS in the myocardium is located in the mitochondria [Becker et al., 1999]. The production of ROS has been shown in isolated hearts and also in isolated cardiomyocytes during ischaemia. The levels of ROS generated during ischaemia are generally low and it is postulated to have an important role in cell signaling.

ROS are produced in large quantities during the first few minutes of post-ischaemic reperfusion and it is generally accepted that these high concentrations of ROS results in oxidative injury. At these high concentrations, ROS may damage myocytes by inducing lipid peroxidation of membranes, thereby altering the integrity, fluidity and permeability of membranes. Another target of ROS is the

membrane proteins, which are involved in ion transport and responsible for maintaining cellular ionic homeostasis [Schulz et al., 2001].

It is now clear that low concentrations of ROS and RNS play a crucial role in signaling pathways necessary for several biological processes, including cardioprotection. It is generally believed that cells are protected against ischaemia/reperfusion damage when they maintain the delicate equilibrium between the protective anti-oxidant signaling and harmful effects of ROS.

It was demonstrated in several studies that ROS production plays an essential role in IPC-induced protection [Baines et al., 1997; Tritto et al., 1997] and it was also postulated that it is the ischaemia-generated ROS that triggers IPC [Van den Hoek et al., 1998]. One of the proposed mechanisms of IPC induced cardioprotection is the opening of the **mitochondrial K<sub>ATP</sub> (mito K<sub>ATP</sub>) channel**, located in the inner mitochondrial membrane [Garlid et al., 2003]. This cardioprotection involves stabilization of the mitochondrial inner membrane and the prevention of mitochondrial uncoupling [Halestrap et al., 2009]. Since 1997, several publications have implicated opening of the mito K<sub>ATP</sub> channel as the main trigger and /or end effector of IPC [Garlid et al., 2003; Gross & Peart, 2003]. Diazoxide has been reported to be a specific activator (opener) of the mito K<sub>ATP</sub> channel and is therefore able to trigger IPC pharmacologically. The fatty acid, 5-Hydroxydecanoate (5-HD), has been reported to be a specific inhibitor of the mito K<sub>ATP</sub> channel [O'Rourke, 2004]. However, diazoxide and 5-HD have various mito K<sub>ATP</sub> channel independent actions and experimental proof that these channels play an essential role in IPC induced protection remains inadequate [Hanley & Daut, 2005].

The ability of ROS to modulate the mito K<sub>ATP</sub> channel has been reported [Forbes et al., 2001; Pain et al., 2000]. ROS primarily formed by mitochondria is superoxide ( $O_2^{\cdot-}$ ) [Murphy et al., 2009], while secondarily generated ROS includes hydrogen peroxide ( $H_2O_2$ ) or lipid peroxides [Kowaltowski et al., 2009]. Both  $O_2^{\cdot-}$  and  $H_2O_2$  have been shown to induce opening of the mito K<sub>ATP</sub> channel [Zhang et al., 2001; Costa et al., 2008]. In addition, it was suggested that opening of the mito K<sub>ATP</sub> channel by  $H_2O_2$  may occur via a PKC- $\epsilon$  dependent mechanism [Costa et al., 2008]. Another source of ROS in the heart is NADPH oxidase and it

is postulated that it may also be involve or contribute to the ROS that trigger IPC. A recent study demonstrated that NADPH almost completely inhibited mito K<sub>ATP</sub> channel activity [Queliconi et al., 2011], suggesting that this channel may play a role in sensing both redox status and energy metabolism [Cardoso et al., 2010]. ROS can also modulate intracellular calcium homeostasis [Feissner et al., 2009]. The transient increase in ROS through the increase in NO levels can trigger both early [Nakano et al., 2000] and late phases [Sun et al., 2005] of ischaemic preconditioning.

### **1.3.2.) Nitric oxide**

Nitric oxide (NO), a simple diatomic gas (structural formula: N=O) and free radical, was originally regarded as an atmospheric pollutant and a component of exhaust fumes and cigarette smoke [Singh & Evans, 1997]. The idea that NO could be endogenously produced in the body was not considered until the concept of “nitrovasodilators” and their role in smooth muscle relaxation was introduced in the early 1980’s [Furchgott & Zawadzki, 1980; Murad, 1998].

The first known physiological interaction described for NO, was the discovery that NO could activate soluble guanylate cyclase (sGC) [Murad 1994; 1998]. Activation of sGC by NO results in the conversion of guanosine triphosphate (GTP) to cyclic monophosphate (cGMP) [Denninger & Marletta, 1999]. This reaction is the trigger of NO’s signaling cascade with cGMP as second messenger, leading to smooth muscle relaxation. NO plays a key role in the regulation of vascular homeostasis.

NO is synthesized by enzymes called nitric oxide synthases (NOS) which are expressed in a variety of tissues. It is particularly highly expressed in the nervous- and cardiovascular system [Schulz et al., 2004]. NOS consists of three known isoforms; neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS or NOS-3). All three isoforms are expressed in the heart [Ballgand & Cannon 1997]. eNOS expression is most prominent in endothelial and cardiac microvascular endothelial cells while iNOS expression has been shown to be relatively higher in cardiomyocytes.

Cardiomyocytes express all three isoforms of NOS. NOS-1 and -3 are constitutively expressed, while NOS-2 is not usually expressed in cardiomyocytes. The expression of NOS-2 is induced during the inflammatory response and is associated with heart failure [Ziolo et al., 2001].

The expression of iNOS is dependent on cytokine stimulation such as TNF- $\alpha$  [Balligand & Cannon, 1997] and it is also known that iNOS generates up to 1000-fold more NO than eNOS [Singh & Evans, 1997]. iNOS derived NO can be potentially harmful due to the sudden, robust generation of NO. It can react with the super-oxide radical ( $O_2^{\cdot-}$ ) leading to the formation of the highly reactive and harmful radical, peroxynitrite ( $ONOO^{\cdot}$ ). The biological effects of NO in the heart depend on which NOS isoform is activated and also the amount of NO released [Strijdom et al., 2009].

NO is regarded as an important mediator of biological processes in the heart and is reported to play an important role in cardioprotection [Boengler et al., 2000]. The sources of NO can both be exogenous or endogenous. Endogenous NO can be generated by activated endothelial NO synthase (eNOS) or upregulated by inducible NOS (iNOS). However, the role of endogenous NO in IPC still remains somewhat controversial [Nakano et al., 2000a]. Results from one study indicated that inhibition of NO synthase attenuates the protective effect of IPC in rats, thereby suggesting a trigger function for nitric oxide in early IPC [Lochner et al., 2000]. Conversely, several other studies indicated that nitric oxide is not a trigger of early IPC [Nakano et al., 2000; Post et al., 2000].

Recent studies however, demonstrated that the cardioprotective properties of NO are partly cGMP dependent [Otani, 2009] and are attributed to the vasodilatory and anti-inflammatory effects of cGMP [Costa et al., 2008]. NO also has direct cardioprotective properties which are cGMP independent. It is also suggested that the direct effects of NO largely target mitochondria. It was further demonstrated that NO can confer protection long after the triggering signal [Boengler et al., 2000]. These studies support the idea that NO works as a signal mediator and end effector of protection, rather than a trigger [Kitakaze 1998; Liu et al., 1991].

Another aspect of NO in late IPC is its dual role as both a trigger and mediator, which involve two different NOS isoforms [Boli, 2001]. It is thought that during the triggering phase of IPC, brief episodes of ischaemia activates eNOS to release NO, which in turn induces the expression of iNOS and a subsequent second burst of NO release [Boli, 2001].

However, the induction of NO synthesis colocalizes with the site where calcium and ROS are exerting their actions. While NO confers many protective functions in the heart, it could be detrimental when simultaneously exposed to excessive oxidative stress. This may result in the generation of further oxidative/nitrosative stress [Costa et al., 2008].

### **1.3.3.) The role of calcium**

Calcium, like ROS serve as a signaling mediator under physiological conditions, but the time period, dose and location are critical [Feissner et al., 2009]. It is widely recognized that intracellular calcium overload causes cellular damage because of ischaemia/reperfusion injury, especially when it is intense and for a prolonged period. However, short and transient exposure to calcium, like ROS, is beneficial because both of them have been reported to trigger IPC [Node et al., 1997; Sun et al., 1996]. Intracellular calcium is required to activate protein kinase C (PKC) during IPC [Node et al., 1997]. Furthermore, the calcium-activated K<sup>+</sup> channel has been reported to protect the myocardium when it is activated at reperfusion [Sato et al., 2005; Shintani et al., 2004].

### **1.4.) Mediators of ischaemic preconditioning**

**Mediators** exert their actions during the period of sustained ischaemia and the first few minutes of reperfusion after sustained ischaemia. Inhibition of a mediator's action during this time will abolish the cardioprotective effect of IPC. Identification and subsequent elucidation of the signaling pathways involved in the cardioprotective effects of mediators in IPC could result in the development of novel pharmacological interventions to be used in clinical therapy.

#### 1.4.1) Protein kinase C (PKC)

The PKC family consists of at least 12 serine/threonine kinases. Many of these PKC isoforms are present in the rabbit heart. The most important PKC-isoforms in the rat heart are the  $\alpha$ -,  $\delta$ - and  $\epsilon$  isoforms. Studies conducted in the rabbit [Ytrehus et al., 1994] and rat [Mitchell et al., 1995] support a central role for PKC as mediator of IPC. Furthermore, studies in different species indicated that PKC epsilon (PKC- $\epsilon$ ) is the main isoform of PKC involved in IPC [Vondriska et al., 2001; Jennings et al., 1997].

The function of PKC- $\delta$  in IPC is controversial. Some studies indicated that it has a protective role in IPC [Kawamura et al. 1998; Mayr et al. 2004], while other studies showed that the expression of PKC- $\delta$  peptides have no protective role in IPC and even worsens ischaemia/reperfusion injury [Chen et al., 2001].

Other researchers also indicated that PKC on its own do not play a role in the protection afforded by IPC, however, simultaneous activation of both PKC and tyrosine kinase play a role in the protective response elicited by IPC [Masaya et al. 2000; Fryer et al.]. It is thus suggested that PKC is not the only mediator of IPC, but that co-mediation with tyrosine kinase (Src and Lck) is likely to occur [Vahlhaus et al.1998]. It is probable that PKC and tyrosine kinase are two components of a much larger kinase cascade as their downstream targets are the mitogen activated protein kinases (MAPK's). The MAPK family is one of the major kinase cascades in the mammalian heart and consists of four families, each of which has been suggested to play a role in cardioprotection (**Discussed in section 1.5**).

Studies demonstrated that PKC is responsible for the generation of IPC- induced activation of 5'-nucleotidase (CD73), which would generate more protective adenosine from adenosine monophosphate [Kitakaze et al., 1995]. It was further proposed that the increase in extracellular adenosine activated the low affinity adenosine A2b receptor, which in turn is protective [Eckle et al., 2007]. According to this hypothesis, IPC hearts should have a higher adenosine concentration than non-IPC hearts. This has been difficult to demonstrate as

most studies did not detect a difference in adenosine concentration between IPC and non-IPC hearts [Schulz et al., 1998].

A minority of studies have shown an increase in adenosine levels in IPC hearts [Eckle et al., 2008], whereas some actually documented lower adenosine levels in IPC hearts [Goto et al., 1996; Harrison et al., 1998]. It was proposed that the activated Gi-PCR concentrate in caveolae during IPC where signalling enzymes are scaffolded into signalosomes which migrate to the mitochondria [Oldenburg et al., 2004]. The signalosomes interact with mitochondria through an unknown PKG mediated mechanism, resulting in the activation of two pools of PKC- $\epsilon$ , 1 and 2 [Costa et al. 2008]. PKC- $\epsilon$ 1 maintains ROS production, while PKC- $\epsilon$ 2 inhibits opening of the mitochondrial permeability transition pore (MPTP). The MPTP is a large conductance pore located in the mitochondria, which opens during reperfusion, resulting in cardiomyocyte death. Closure of the MPTP during reperfusion is cardioprotective (**discussed in section 1.6.2**).

#### **1.4.2.) Sphingokinase**

The activation of sphingokinase in the setting of IPC has been demonstrated to be dependent on the activation of PKC- $\epsilon$ . Sphingosine 1-phosphate, a lipid signalling molecule, generated by sphingokinase is implicated as a potential mediator of IPC-induced protection [Jin et al., 2004].

#### **1.4.3.) Protein kinase A (PKA)**

Protein kinase A (PKA) is a ubiquitous cellular kinase that phosphorylates serine and threonine residues in response to cAMP [Niswender et al., 2002]. The widespread expression of PKA subunits, together with the numerous mechanisms by which cAMP is regulated within the cell, suggests that the PKA signaling pathway is of importance in cellular function. PKA is a key regulatory enzyme in the catecholamine-mediated control of excitation-contraction coupling in the heart. It also plays an important role in the activation of transcription factors as well as the control of metabolic enzymes. The PKA enzyme is composed of two catalytic (C $\alpha$  and C $\beta$ ) and one regulatory (R) subunit [Corbin et al., 1988]. Activation of the regulatory subunit by cAMP results in the release of the catalytic

subunits from the PKA holoenzyme. The free catalytic subunits (C $\alpha$  and C $\beta$ ) can subsequently function as active holoenzymes. The C $\alpha$  and C $\beta$  subunits are believed to have different functions and have been shown to phosphorylate different targets [Yu et al., 2004].

The important role of  $\beta$ -adrenergic receptor stimulation and possible involvement of PKA in the pathogenesis and treatment of heart failure is well accepted [Lohse et al., 2003]. Excessive  $\beta$ -adrenergic receptor stimulation in heart failure can lead to adverse effects on myocardial function as has been demonstrated in the setting of ischaemia/reperfusion [Waldenstrom et al., 1978; Rona, 1985]. Ischaemia/ reperfusion in the heart is characterized by cAMP accumulation, accompanied by activation of PKA [Sakai et al., 1999], increased phosphorylation and opening of L-type calcium channels, resulting in the development of cytosolic calcium overload [Shine & Douglas, 1983; Du Toit & Opie, 1992].

The role of PKA in the cardioprotection afforded by IPC is controversial. A study by Makaula and coworkers (2005) demonstrated that inhibition of PKA before sustained ischaemia enhanced the cardioprotection induced by IPC. These results suggest a harmful role for PKA in this regard.

In contrast, it was demonstrated in a different study that intramyocardial accumulation of cAMP mimicked the cardioprotective effect of IPC. Inhibition of PKA attenuated the protective effect of IPC, suggesting a mediator function for PKA in the setting of IPC [Lochner et al., 1999]. Both elevated levels of cAMP and activation of PKA were shown to be independent of PKC [Sanada et al., 2001; Sato et al., 2005]. It was reported that instead of PKC, the activation of p38 could be involved as a downstream mechanism of protection [Sanada et al., 2001]. It was also reported that Rho kinase play an important protective role downstream of PKA during sustained ischaemia [Sanada et al., 2004]. Importantly, apart from its protective effects, the RhoA/Rho-kinase mediated pathway plays a key role in vascular smooth muscle contraction, cell adhesion and motility, cytokinesis and gene expression. All of these processes are involved in the pathogenesis of atherosclerosis [Shimokawa, 2002].



#### **1.4.4.) Protein kinase G (PKG)**

Protein kinase G (PKG) is a cGMP dependent serine/threonine protein kinase and forms part of the NO-sGC-cGMP pathway. This pathway is regarded as the predominant molecular mechanism of the actions of NO [Balligand & Cannon 1997]. Activation of the NO-sensitive sGC generates cGMP from GTP, resulting in the activation of two cGMP-dependent protein kinases (PKG 1 and PKG II) [Schultz et al., 2004]. Phosphodiesterase II (PDE II) and phosphodiesterase III (PDE III) are second class molecular targets of cGMP. PDE II is activated by cGMP and PDE III is inhibited by cGMP [Schultz et al., 2004]. cGMP have also been demonstrated to regulate the activity of cGMP-regulated ion channels [Friebe & Koesling, 2003]. Together, these effectors are implicated in the regulation of a number of important physiological functions in the cardiovascular system.

PKG was first implicated as a potential mediator of IPC, when studies indicated that cGMP levels were increased in IPC hearts [Iliodromitis et al., 1996]. Work done in Downey's laboratory (2005) suggested that PKG- $\epsilon$  can mediate opening of the mito  $K_{ATP}$  channel during IPC-induced cardioprotection [Costa et al., 2005]. The mechanism of this activation was unknown, but it was suggested that ROS may be involved, since direct activation of PKG resulted in the production of ROS. It was also reported that NO, guanylate cyclase, cGMP and PKG are the key role players in activation of the mito  $K_{ATP}$  channels. This protection elicited by the NO/PKG signaling pathway was associated with a decrease in LDH release and DNA damage. The protective effects induced by the NO/PKG pathway were blocked by the two inhibitors of the mito  $K_{ATP}$  channel, glibenclamide and 5-HD. These results suggested that NO, PKG and the mito  $K_{ATP}$  channel contribute to the mechanism involved in IPC induced cardioprotection [Cuong et al., 2005].

#### **1.5.) Mitogen Activated Protein Kinases (MAPK's)**

The MAP Kinase (MAPK) family is one of the major kinase cascades in the mammalian heart. MAPKs regulate diverse cellular processes including gene expression, differentiation, motility, mitosis, differentiation, metabolism, cell survival and apoptosis [Roux & Blenis, 2004]. MAPKs are serine/threonine

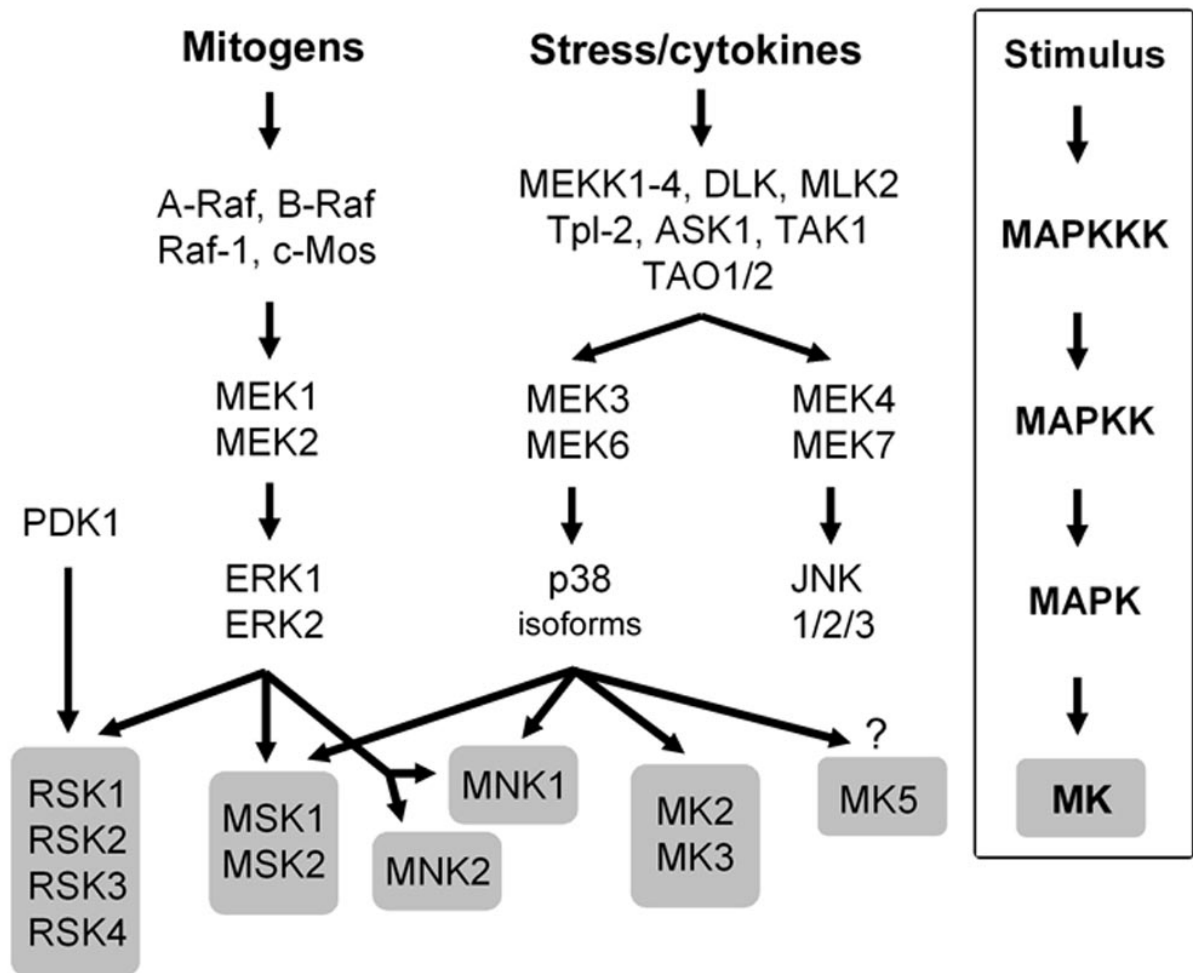
kinases which require dual phosphorylation on a Thr- and Tyr motive for activation [Boulton et al., 1990]. All eukaryotic cells possess multiple MAPK pathways of which the most extensively studied groups are the 44 and 42-kDa extracellular signal-regulated kinase family (ERK p44 and ERK p42 MAPK), Big Map Kinase 1 (BMK 1/ERK 5) and the two stress-activated MAPK families: the 46- and 54-kDa c-Jun-NH<sub>2</sub> amino- terminal kinase (JNK) and the 38 kDa, p38 MAP Kinase family [reviewed by Roux & Blenis, 2004] (**Figure 1.2**).

#### **1.5.1.) p44/p42 MAP Kinases (ERK 1 and 2)**

The mammalian ERK 1 and 2 cascade is known as the classical mitogen activated protein kinase cascade and comprises of the MAPKKKs: A-Raf, B-Raf and Raf-1; the MAPKKs, MEK1 and MEK2 and the MAPKs, ERK1 and ERK2 (**Figure 1.2**). ERK 1 and ERK 2 have 83% amino acid homology and are expressed to various extents in quiescent cells in all tissues [Chen et al., 1992]. ERK 1 and 2 are potently stimulated by growth factors, serum and phorbol esters, but are minimally activated by ligands of heterotrimeric G-protein coupled receptors, cytokines, osmotic stress and microtubule disorganization [Lewis et al., 1998].

The observations that several growth factors with anti-apoptotic effects [Buerke et al., 2001], such as fibroblast growth factor [Htun et al., 1998], insulin-like growth factor [Buerke et al., 2001] and cardiotrophin-1 [Kuwahara et al., 2000] can activate ERK, suggested a cardioprotective role for ERK in cell survival. A major cardioprotective role for ERK p42 was demonstrated in opioid-induced cardioprotection in rats [Fryer et al., 2001]. It was indicated in several models that ERK signaling was activated during ischaemia in the rat [Miyakawa et al., 2001], *in vivo*, in a pig model [Baracik et al., 1997] and in neonatal rat cardiomyocytes [Ye et al., 2000].

It was also reported in several studies that ERK activation is induced during ischaemia/reperfusion in rat, bovine, human and guinea pig hearts [Araujo et al., 2001; Talmor et al., 2000], while other studies, on the other hand, failed to indicate involvement of ERK in the setting of ischaemia/reperfusion [Bogoyevitch et al., 1996; Clerk et al., 1998].



**Figure 1.2:** Signaling cascades leading to activation of the MAPK-activated protein kinases (MAPKs). Mitogens and cellular stresses lead to activation of the ERK1/2 and p38 cascades, which in turn phosphorylate and activate the five subgroups of MKs. MKs represents an additional enzymatic amplification step in the MAPK catalytic cascades. See text for details [Roux & Blenis, 2004]

Furthermore, it was also demonstrated by numerous studies that various MAPK cascades are involved in the setting of ischaemic preconditioning (IPC) - induced cardioprotection [Cohen et al., 2000]. In this context, activation of the ERK pathway was demonstrated after IPC, in an isolated working rat heart model. [Maulik et al., 1996]. The IPC protocol consisted of four cycles of 5 min ischaemia followed by 10 min reperfusion. Normothermic ischaemia was induced for 30 min, followed by 30 min reperfusion. ERK was activated after 30 min reperfusion. The contribution of ERK activation to protection was not tested in this study [Maulik et al., 1996]. Activation of ERK during IPC was also reported in an in vivo rat heart model [Fryer et al., 2001]. IPC was induced by one cycle of a 5 min coronary artery occlusion followed by 5 min reperfusion. Activation of ERK was increased during IPC after 5 min, 30 and 60 min of reperfusion compare to control. The ERK blocker, PD98059 abolished IPC-induced protection and activation of ERK [Fryer et al., 2001]. A study by Ping and co-workers [1999], utilizing the conscious rabbit model of IPC, demonstrated activation of ERK directly after the IPC protocol. The IPC protocol consisted of 6 cycles of 4 min coronary occlusion followed by 4 min reperfusion. This activation of ERK was completely blocked by the PKC inhibitor chelerythrine, suggesting that PKC activates the ERK pathway in IPC [Ping et al., 1999]. A study by Hausenloy and co-workers [2005] demonstrated a biphasic activation of ERK in a rat heart Langendorff perfusion model with regional ischaemia. IPC hearts were subjected to two 5 min periods of global ischaemia followed by 10 min reperfusion, before 35 min of regional ischaemia. This was followed by 120 min reperfusion after which infarct size was measured. ERK was activated after the IPC stimulus and the second phase of ERK activation occurred after 120 min reperfusion. Inhibition of ERK activation with the MEK1/2 inhibitor PD98059 abrogated IPC-induced protection.

The role of ERK in IPC was further emphasized when it was demonstrated that PKC- $\epsilon$  may act as an upstream activator of ERK 1 and 2 [Ping et al., 1999]. In addition, it was suggested that the generation of ROS in response to IPC is able to activate ERK 1 and 2 [Samavati et al., 2002]. The translocation of ERK 1 and 2 to the nucleus [Ping et al., 1999], the intercalated discs [Da Silva et al., 2004], the cytosol [Fryer et al., 2001] and the mitochondria [Baines et al., 2002] were demonstrated after a preconditioning stimulus. ERK 1 and 2 has been demonstrated to activate NF- $\kappa$ B and AP-1 transcription factors in the nucleus

[Ping et al., 1999]. These transcription factors are implicated in delayed IPC. ERK 1 and 2 can activate MAPKAP kinase 2  $\alpha$  and  $\beta$ , leading to phosphorylation of the small heat shock protein 2, which is important in stabilizing the integrity of the cytoskeleton during ischaemia, thus preserving cell viability [Chevalier & Allen, 2001]. ERK 1 and 2 can phosphorylate p90RSK and inhibit GSK-3 $\beta$  leading to the inhibition of MPTP opening and cardioprotection [Eldar-Finkelman et al., 1995; Frodin et al., 1999]. It was also suggested that hypoxia inducing factor-1 $\alpha$  (HIF-1 $\alpha$ ) may be a potential downstream target of ERK 1 and 2 in IPC [Liu et al., 2003]. HIF-1 $\alpha$  is activated in response to hypoxia and is important in the regulation of genes concerned with cellular survival during hypoxia [Cai et al., 2003].

Although the majority of studies support a role for ERK in IPC, the role of ERK as a potential mediator of IPC has been controversial because several studies failed to demonstrate any activation of ERK during IPC. Kim and co-workers [1999], demonstrated ERK activation after post-ischaemic reperfusion in control as well as IPC hearts. IPC was induced in rabbit hearts with 5 min ischaemia followed by 10 min reperfusion. Hearts were subjected to 30 min regional ischaemia followed by 2 hours reperfusion. Infarct size was measured as endpoint of functional recovery. In the IPC hearts infarct size was reduced after 2 hours reperfusion, compare to controls. Infusion of the MEK1/2 inhibitor PD98059 during ischaemia, however, did not abrogate IPC-induced protection. These results demonstrated that a reduction in infarct size by IPC was independent form ERK activation. A study by Mocanu and co-workers [2002], demonstrated that ERK was phosphorylated in response to IPC, but is not required for IPC-induced protection. In this study, rat hearts were preconditioned with two cycles of 5 min ischaemia and 10 min reperfusion on a Langendorff perfusion system. Hearts were subjected to 35 min regional ischaemia, followed by 120 min reperfusion prior to infarct size assessment. Administration of the MEK inhibitor PD98059 during the IPC protocol had no effect on the reduction of infarct size, indicating that the ERK signaling pathway is not implicated in IPC [Mocanu et al., 2002]. Takeishi and co-workers [2001] failed to demonstrate a role for ERK as mediator of IPC. Guinea pig hearts were perfused on a Langendorff apparatus with a saline-filled latex balloon attached to a catheter inserted into the left ventricle, through the mitral valve for pressure measurements. Hearts were preconditioned by 5 min global

ischaemia followed by 10 min reperfusion, prior to 20 min global ischaemia. Takeishi and co-workers [2002] could not detect any IPC-induced ERK activation during ischaemia and reperfusion in this study, suggesting that ERK may not play a role in IPC. Mockridge and co-workers [2000] also failed to demonstrate a role for ERK as mediator of IPC in a cardiomyocyte model. Cardiomyocytes from rats were preconditioned and reperfused for 24 hours in this study. Although ERK was activated during reperfusion with maximum activation at 10 min reperfusion, infusion of the MEK inhibitor PD98059 30 min before IPC did not block cardioprotection. These results suggest that the protective effect of IPC was independent of ERK activation [Mockridge et al., 2000].

### **1.5.2.) p38 MAPK**

The p38 family consists of four isoforms, p38- $\alpha$ , p38- $\beta$ , p38- $\gamma$  and p38- $\delta$  of which the  $\alpha$ - and  $\gamma$  isoforms are the most abundant in the myocardium [Saurin et al., 2000]. p38 has two activation sites, a threonine residue at amino acid 180 and a tyrosine residue at site 182. Both activation sites must be phosphorylated for full activation to occur. p38 is activated by a MAP kinase kinase (MEK 3 or MEK 6), which itself has to be activated first by a MAP kinase kinase kinase (MAPKKK).

It has been shown that the different isoforms of p38 mediate different biological functions [Michel et al., 2001]. In neonatal rat cardiomyocytes, p38- $\beta$  is anti-apoptotic, while p38- $\alpha$  mediates apoptosis [Wang et al. 1998]. Several studies demonstrated that p38 is phosphorylated within minutes during regional no-flow ischaemia and global ischaemia in isolated rat hearts [Bandyopadhyay et al., 1997; Bogoyevitch et al., 1996]. This was also found in dog, pig and rat hearts *in vivo*.

The activation of p38 during prolonged ischaemia is transient and the phosphorylation of p38 may be rapidly reduced towards pre-ischaemic values after prolonged ischaemia. Following this reduced activation of p38, it is once again increased upon reperfusion [Yin et al., 1997; Shimizu et al., 1998].

It was demonstrated that both PKC and tyrosine kinases are located upstream of p38 in the IPC signaling pathway, suggesting that they may act as activators of p38 [Weinbrenner et al., 1997].

Potential downstream targets of p38 in IPC are myofilaments, the sarcolemma and also the nucleus [Da Silva et al., 2004]. Other studies have shown that p38 forms signaling modules with PKC- $\epsilon$  at the level of the mitochondria in the setting of IPC [Baines et al., 2002]. Recent studies indicated co-localization of p38 with connexin-43. This was associated with an increase in p38 activation, suggesting that p38 may protect by reducing gap junction permeability [Schulz et al., 2003].

MAPKAP kinase 2 is an important substrate and target of p38. p38 has been shown to phosphorylate MAPKAP kinase 2, which in turn phosphorylates the small HSP-27, which protect cardiomyocytes against ischaemic damage [Rouse et al., 1994]. HSP-27 is highly abundant in cardiac and skeletal muscles and associates with actin [Efthymiou et al., 2004]. It is also vital to muscle assembly [Brown et al., 2007]. HSP-27 increases in response to stress to protect against insults such as ischaemia through stabilization of the actin cytoskeleton. HSP-27 protects against ischaemia/reperfusion damage where it plays a key role in anti-oxidant mechanisms. Studies have shown that hypoxic preconditioning resulted in translocation of HSP-27 from the cytosol to the cytoskeleton. This translocation was dependent on p38 activation [Armstrong et al., 1999].

Although the p38 signalling pathway in IPC has been extensively studied, published observations are inconsistent. A majority of studies reported activation of p38 during the triggering phase of IPC, while inhibition of p38 during this phase of IPC substantially attenuated the protective effect of IPC as indicated by an increase in infarct size. These results imply a major role for p38 activation during the triggering phase in IPC induced protection [Nakano et al., 2000; Sakamoto et al., 2000 and Sanada et al., 2001]. The study by Nakano and co-workers [2000] investigated p38 activation in isolated rabbit hearts. The Langendorff perfusion system was used with infarct size as endpoint. IPC was induced by a single cycle of 5 min ischaemia, followed by 10 min reperfusion prior to 20 min global ischaemia. These results demonstrated that IPC activates p38 during the triggering phase of IPC. The potent blocker of p38, SB203580, blocked the



protection afforded by IPC as measured by infarct size [Nakano et al., 2000]. The study by Sakamoto and co-workers [2000], used the Langendorff perfusion model with a latex balloon inserted in the left ventricle connected to a pressure transducer to monitor the heart rate and left ventricular pressure (LVP). Rat hearts were preconditioned by a single cycle of 5 min ischaemia, followed by 5 min reperfusion. Subsequently hearts were subjected to 40 min global ischaemia, followed by 50 min reperfusion. These results demonstrated p38 activation during the triggering phase of IPC to be protective as blockade with SB203580 inhibited IPC-induced protection [Sakamoto et al., 2000]. The study by Sanada and co-workers [2001], utilized an *in vivo* canine heart model with infarct size as endpoint to investigate p38 activation. IPC was induced by 4 cycles of 5 min coronary occlusion, followed by 5 min reperfusion. Subsequently the coronary artery was occluded for 90 min (sustained ischaemia) followed by 6 hours of reperfusion. These results demonstrated p38 activation during the triggering phase of IPC. Inhibition of p38 during IPC using SB203580, completely reverses the infarct size limitation by IPC, suggesting that activation of p38 during this phase is protective [Sanada et al., 2001].

On the other hand, several studies indicated that the activation of p38 during the sustained ischaemic period was increased after IPC and that p38 may act as a mediator of IPC [Schulz et al., 2002; Nakano et al., 2000; Weinbrenner et al., 1997 and Eaton et al., 2002]. Schulz and co-workers [2002], utilized an *in vivo* swine heart model of IPC. Pig hearts were preconditioned by 10 min ischaemia followed by 15 min reperfusion, prior to 90 min sustained ischaemia followed by 120 min reperfusion. These results demonstrated that p38 was phosphorylated during sustained ischaemia in the IPC hearts. Blockade of p38 with SB203580 before sustained ischaemia attenuated the infarct size reducing effect of IPC [Schulz et al., 2002]. The study by Nakano and co-workers [2000] utilized an isolated rabbit heart model, using the Langendorff perfusion system with infarct size as endpoint. IPC was induced by 5 min ischaemia, followed by 10 min reperfusion prior to 20 min global ischaemia. It was demonstrated that IPC activates p38 and also its downstream substrate MAPKAP2 during the sustained ischaemic period in IPC hearts. The p38 inhibitor SB203580, blocked the protection afforded by IPC as measured by infarct size, suggesting that p38 is a mediator of IPC in this model [Nakano et al., 2000]. Weinbrenner and co-workers



[1997] investigated p38 activation in an isolated rabbit heart model. The Langendorff perfusion system was used with a saline filled latex balloon inserted into the left ventricle. IPC was induced by 5 min global ischaemia, followed by 10 min reperfusion, before the onset of 30 min global ischaemia. It was demonstrated that IPC activated p38 after 10 and 20 min global ischaemia. The p38 blocker SB203580, attenuated the protective effect of IPC and also the activation of p38 during sustained ischaemia. These results suggest a mediator function for p38 in IPC [Weinbrenner et al., 1997]. Eaton and co-workers [2000] utilized the Langendorff perfused rat heart model of IPC. Ventricular function was assessed via a latex balloon which was inserted in the left ventricle connected to a pressure transducer. IPC was induced by three cycles of 5 min ischaemia ; followed by three cycles of 5 min reperfusion, prior to 20 min ischaemia and 60 min reperfusion. p38 Activation was increased during the 20 min ischaemic period. Protection by p38 activation was not tested for by infusion of the p38 blocker.

*In vitro* studies indicated that IPC primarily prevents the activation of p38- $\alpha$  during sustained ischaemia in p38  $\alpha$ -transfected rat cardiomyocytes. p38- $\beta$  was shown to be downregulated during sustained ischaemia in control and ischaemic preconditioned cells [Saurin et al., 2000]. IPC appears to require activation of the p38- $\alpha$ , but not the p38- $\beta$  isoform [Sicard et al., 2010]. However other studies failed to show any contribution of p38 towards IPC [Ping et al., 1999; Barancik et al., 2000; and Gysembergh et al., 2001]. Ping and co-workers [1999] utilized an *in vivo* rabbit heart model of IPC. IPC was induced by 6 cycles of 4 min coronary occlusion followed by 4 min reperfusion. This study failed to show any contribution of p38 towards IPC-induced protection [Ping et al., 1999]. Barancik and co-workers [2000] investigated p38 activation in an *in vivo* swine heart model of IPC. The IPC protocol consists of 2 cycles of 10 min ischaemia followed by 10 min reperfusion, prior to 40 min index ischaemia and 60 min reperfusion. The p38 blocker SB203580 was administered 15 min before IPC and also during the IPC protocol. Inhibition of p38 did not influence the infarct size reduction by IPC, suggesting that p38 does not contribute to IPC-induced protection [Barancik et al., 2000]. The study by Gysembergh and co-workers [2001] utilized an isolated rabbit heart model of IPC. The Langendorff perfusion system was used with a balloon inserted in the left ventricle. IPC was induced by 2 cycles of 5 min

episodes of antecedent PC ischaemia. This study failed to show any contribution of p38 towards IPC-induced protection [Gysembergh et al., 2001].

Studies showing attenuation in p38 activation during the sustained ischaemic period after the IPC stimulus, suggest a detrimental role for p38 activation during sustained ischaemia [Kimura et al., 2005; Saurin et al., 2000; Rakhit et al., 2001; Fryer et al., 2001]. This finding was consistent with numerous other studies showing that inhibition of p38 during sustained ischaemia is protective against reperfusion damage [Mackay et al., 1999; Ma et al., 1999; Kimura et al., 2005]. Therefore, the role of p38 as a mediator of protection in IPC remains controversial and since it has a major role in the triggering phase, it was suggested to act as a trigger rather than a mediator in IPC [Maulik et al., 1998]. The importance of p38 activation in the setting of cardioprotection thus remains controversial.

### 1.5.3.) JNK

JNK 1, JNK 2 and JNK 3 (also known as SAPK $\alpha$ , SAPK $\beta$  and SAPK $\gamma$  respectively), also known as stress activated protein kinases (SAPK) are ubiquitously expressed [Kyriakis et al., 1994]. JNK 3 is primarily expressed in the brain, whereas JNK 1 (46 kDa) and JNK 2 (54 kDa) are expressed in the heart. In contrast to ERK 1 and 2, the JNK's are strongly activated by cellular stresses such as heat, osmotic shock, UV irradiation, endotoxins and cytokines and to a lesser extent by serum and growth factors [Kyriakis & Avruch, 1990]. JNK requires dual phosphorylation on tyrosine and threonine residues within a conserved Thr-Pro-Tyr motif and are activated by the upstream MAPKKs, MKK4 and MKK7, which are activated by several MAPKKKs, including MKK1-4, MLK2 and -3, TPL-2, DLK, TAO1 and -2, TAK 1, ASK 1 and -2 [Kyriakis & Avruch, 1990] (**Figure 1.2.**). MKK7 is capable of activating both p38 as well as JNK, whereas MKK4 is specific for JNK. Both JNK isoforms are significantly activated upon reperfusion, but are not affected by ischaemia alone.

Studies have demonstrated that ROS mediated the activation of JNK at reperfusion [Knight et al., 1996]. Others have indicated that hypoxic preconditioning-induced activation of JNK is dependent on PKC- $\epsilon$  [Ping et al.,

1999]. JNK can also be activated by Gq-coupled receptors and subsequent activation of PKC.

Once activated, JNK rapidly phosphorylates the transcription factor c-Jun within its N-terminal domain. It was also reported that overexpression of PKC- $\epsilon$  activated the transcription factors NF- $\kappa$ B and AP-1, which are important mediators of delayed IPC - this was shown to be dependent on the activation of JNK [Li et al., 2000]. Over-expression of active PKC- $\epsilon$  in murine myocytes resulted in the formation of signalling modules, comprising JNK, ERK 1 and 2 and also p38, at the level of the mitochondria [Baines et al., 2002]. It is possible that the brief episodes of ischaemia/reperfusion, as seen in IPC, could activate the JNK pathway. Some studies suggested a potential role for JNK as mediator of protection in IPC. Activation of JNK 46 during ischaemia was reported to be mediated by PKC. Inhibition of JNK 46 with curcumin blocked the infarct size reduction of IPC in isolated rat heart, thereby supporting a mediator function in IPC. Other studies could not confirm the mediator function of JNK in IPC induced protection. Several studies however, indicated activation of JNK during the triggering phase of IPC [Haq et al., 1998], suggesting that JNK mediates the protective effect of IPC [Sato et al., 2000]. Some studies however, demonstrated JNK activation in response to an IPC stimulus but failed to indicate a contribution to protection [Iliodromitis et al., 2002]. Therefore the role of JNK in IPC remains controversial.

#### **1.5.4.) Phosphatidylinositol 3- kinase (PI3-K) and PKB/Akt**

Phosphoinositide 3-kinase (PI3-Ks) forms part of a unique family of enzymes with the ability to function as both lipid- as well as protein kinases. Based on their substrate specificity, molecular structure and mode of action, mammalian PI3-Ks are divided into three classes (I, II and III) [Oudit et al., 2004]. Upon activation PI3-Ks phosphorylate the plasma membrane lipid phosphatidyl-inositol-4,5-bisphosphate (PIP<sub>2</sub>) to form phosphatidyl-inositol-3,4,5- triphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> acts as a second messenger by recruiting a variety of different downstream components such as phosphoinositide-dependent kinase-1 (PDK1) and phosphoinositide-dependent kinase-2 (PDK2) which phosphorylates and activates various protein kinases [Burgering & Coffey, 1995]. Akt, also known as

Protein Kinase B (PKB), is one of the targets of the phosphatidylinositol 3-kinase (PI3-K)-related signalling pathway.

Class I PI3-K can be divided into two subclasses, namely Class I A PI3-K and Class 1B PI3-K, which are the main isoforms expressed in cardiomyocytes [Naga Prasad et al., 2003]. Class I PI3-K's are heterodimers of a 110-kDa catalytic subunit (p110- $\alpha$ , p110- $\beta$  and p110- $\delta$ ) and a regulatory subunit of 85 or 55 kDa (p85/p55). Class I PI3-Ks are stimulated by receptor tyrosine kinase cascades and modulate cardiac growth [Luo et al., 2005].

Class 1B PI3-K consists of a p110 $\gamma$  catalytic subunit and a p101 regulatory subunit. It has been shown to couple to GPCR and has the ability to modulate cAMP metabolism in ventricular myocytes and is an important regulator of cardiac contractility [Patrucco et al., 2004; Crackower et al., 2002]. In addition, PI3-K- $\gamma$  has been shown to play a critical role in the induction of fibrosis, cardiac hypertrophy and cardiac dysfunction as a consequence of long term  $\beta$ -AR stimulation [Oudit et al., 2003].

The serine/threonine protein kinase PKB/Akt is the main effector of the PI3-K-related signalling pathway and plays a central role in the regulation of cellular growth, survival and metabolism across many species [Jian-Zhi et al., 2012]. PKB/Akt exists as 3 isoforms (Akt-1, Akt-2 and Akt-3) in the mammalian heart. The Akt-1 isoform predominates in the heart and it has an important role in protecting the myocardium against ischaemia/reperfusion injury [Shiraishi et al., 2004]. Expression of Akt-2 also occurs in the heart, whilst Akt-3 phosphorylation is upregulated in diseased hearts [Taniyama et al., 2002]. PKB can be phosphorylated at two sites (serine 473 and threonine 308) [Alessi et al., 1996]. It has been suggested that full activation of PKB requires phosphorylation at both sites, therefore it requires two phosphorylation events [Tsurutani et al., 2006].

PKB/Akt is activated in response to growth factors and hormones including insulin, insulin growth factor-1 (IGF-1), as well as through  $\beta$ -adrenergic stimulation and plays an important role in the regulation of cardiac hypertrophy, angiogenesis and apoptosis [Oudit et al., 2004]. Activated PKB results in a signal

transduction cascade, leading to the inhibition of apoptosis and thereby promoting cell survival [Davidson et al., 2006].

It was first demonstrated in 2000 [Tong et al., 2000] that IPC protects the heart by activating PI3-K-Akt. This study was conducted in a Langendorff perfused rat heart model of IPC. A latex balloon connected to a pressure transducer was inserted in the left ventricle to monitor left ventricular developed pressure (LVDP). IPC was induced by 4 cycles of 5 min ischaemia and 5 min reperfusion. The PI3-kinase inhibitor wortmannin (WM) was administered for 5 min before and throughout IPC. It was demonstrated that WM treatment blocked the protective effect of IPC and also the IPC-induced increase in PKB/Akt [Tong et al., 2000]. This finding was confirmed in subsequent studies, suggesting that Akt may act as a mediator of IPC induced protection. Mocanu and co-workers [2002] investigated the activation of PKB/Akt in an isolated rat heart Langendorff perfusion model. A latex balloon was inserted in the left ventricle to monitor LVDP. The IPC protocol consisted of two cycles of 5 min global ischaemia, followed by 10 min reperfusion, prior to 35 min regional index ischaemia and 120 min reperfusion. The PI3K inhibitor, wortmannin (WM) was administered throughout the IPC protocol. It was demonstrated that blockade of IPC with WM resulted in only partial attenuation of protection [Mocanu et al., 2002]. In a study by Hausenloy and co-workers [2005], the isolated rat heart Langendorff model of IPC was used with functional recovery as endpoint. IPC was induced by two 5 min periods of global ischaemia, followed by 10 min reperfusion, prior to 35 min regional ischaemia and 120 min reperfusion. The PI3K inhibitor LY294002 was infused into the perfusate for the first 15 min of reperfusion. IPC induces a biphasic response in PKB/Akt phosphorylation. The first phase occurred immediately after the IPC stimulus, whereas the second phase occurred at reperfusion. The PI3K inhibitor attenuated this biphasic activation of PKB/Akt and abrogate the IPC mediated reduction in infarct size [Hausenloy et al., 2005].

However, several other studies failed to demonstrate a role for Akt as a mediator of IPC [Budass et al., 2006; Lecour 2009]. Some studies failed to show any contribution of PKB/Akt towards IPC-induced protection. Button and co-workers investigated PKB/Akt activation in adenosine receptor mediated IPC. Right ventricular strips were isolated from adult wistar rats and mounted between

platinum electrodes and isometric force transducers. Hypoxic IPC was induced by 10 min hypoxia, followed by 20 min reoxygenation prior to 30 min hypoxia followed by 30 min reoxygenation. Wortmannin (WM) was added 30 min before hypoxic IPC or treatment with adenosine receptor agonist and removed before 30 min hypoxia. It was demonstrated that although the selective adenosine receptor agonist induced IPC in the right ventricular strips, the effect is independent of PI3K- PKB/Akt dependent signaling pathways [Button et al., 2005]. Germack and co-workers [2005] investigated the activation of PKB/Akt in neonatal ventricular myocytes prepared from 1-4 days old Wistar rats. Cells were pre-treated for 15 min with adenosine before 4 hours of hypoxia followed by 18 hours of reoxygenation. Cell viability was assessed using the sodium 3' -[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate assay (XTT assay) and Lactate dehydrogenase (LDH) release. The PI3K inhibitor Wortmannin (WM) was administered 15 min before adenosine treatment, followed by 4 hours of hypoxia. WM did not reverse the cardioprotection of adenosine IPC, suggesting that the PI3K-PKB/Akt pathway is not involved [Germack et al., 2005].

Tyrosine kinase is a known upstream activator of PI3-K-Akt [Cantley et al., 2002]. Studies have demonstrated that the tyrosine kinase Src is located upstream from PI3-K-Akt activation in the IPC signaling pathway [Krieg et al., 2002]. It was further demonstrated that both eNOS and PKC- $\epsilon$  are located downstream of Akt in the IPC signalling pathway. This finding has been confirmed by other studies [Krieg et al., 2003 & 2004; Costa et al., 2005]. PI3-K /Akt are reported to induce NO production upon IPC and reperfusion [Tong et al., 2000].

The majority of the downstream targets phosphorylated by PKB/Akt activate various anti-apoptotic pathways [Hausenloy et al., 2004]. It is suggested that PKB/Akt may protect the myocardium against ischaemia/reperfusion injury, by acting at the level of the mitochondria, through the inhibition of GSK-3 $\beta$  and subsequent inhibition of the mitochondrial permeability transition pore [Davidson et al., 2006].

However, chronic activation of the PKB/Akt pathway may lead to cardiac hypertrophy. It appears that a fine balance has to be maintained between the

potential beneficial effects of activating this pathway and the potentially detrimental effects of sustained activation of this pathway [Franke et al., 2003].

#### **1.5.5.) The involvement of PKB/Akt and ERK p44/p42 MAPK in cardioprotection**

Lethal reperfusion injury or ischaemia/reperfusion damage specifically refers to the component of cell death occurring as a consequence of reperfusion, following a transient period of ischaemia [Piper et al., 1998]. However, it is well established that activation of the prosurvival kinases, PKB/Akt and ERK p44/p42 MAPK a.k.a. the Reperfusion Injury Salvage Kinase (RISK) pathway, at the onset of reperfusion, lead to myocardial protection against ischaemia/reperfusion (I/R) damage [Hausenloy & Yellon, 2004]. An earlier study by Hausenloy and co-workers, utilizing the isolated perfused rat heart model, indicated that IPC results in phosphorylation of both Akt and ERK 1 and 2 after 15 min reperfusion. Inhibition of either PKB or Akt activation at 15 min reperfusion abolished protection, indicating that phosphorylation of both these kinases was essential to mediate the protection induced by IPC [Hausenloy et al., 2003].

A later study reported that signaling through the PKB/Akt and ERK p44/p42 pathways can mediate cellular survival by an anti-apoptotic mechanism. This is achieved by phosphorylation and subsequent inactivation of a distal target, the pro-apoptotic protein BAD [Inamura et al., 2010].

Investigation into the downstream targets of PI3-K has focused on the role of GSK-3 $\beta$  inhibition in the cardioprotection by ischaemic preconditioning [Tong et al., 2002]. GSK-3 $\beta$  is inactivated through phosphorylation by PI3-K and is a critical downstream mediator of IPC. This kinase serves as a downstream effector of the Wnt/Frizzled pathway-induced cardioprotection [Barandon et al., 2005].

The mitochondrial outer membrane (OM) contains a channel protein known as the voltage-dependent anion channel (VDAC). It is the most abundant protein of the OM and allows non-specific small substances of less than 5 kDa into the intermembrane space and cytosol. Opening of VDAC is a regulated process,



since the channel exhibit some degree of specificity in the mitochondrial import/export of molecules such as ATP, calcium and other ions. The role of VDAC in apoptosis was first demonstrated by Shimizu and co-workers in 1999. Results from this study demonstrated that the pro-apoptotic proteins Bax and Bak accelerate opening of VDAC. This was accompanied by cytochrome c release from the mitochondria and Bax/Bak-induced loss of mitochondrial membrane potential [Shimizu et al., 1999]. A later study by the same group demonstrated that the anti-apoptotic proteins Bcl-2 and Bcl-XL, prevented VDAC opening and cytochrome c release [Shimizu et al., 2001]. Taken together, the results from these studies suggest that the Bcl-2 family of proteins regulate the mitochondrial membrane potential and cytochrome c release from the mitochondria, through binding to VDAC to inhibit apoptosis.

The mitochondrial inner membrane is less permeable and allow only small molecules like H<sub>2</sub>O, O<sub>2</sub>, CO<sub>2</sub> or NH<sub>3</sub> to enter/exit. The mechanism that induces the release of larger molecules by the mitochondria, such as cytochrome c, is known as the mitochondrial permeability transition (MPT). It is proposed that the mitochondrial permeability transition pore (MPTP) is composed of adenine nucleotide translocase, a mitochondrial phosphate carrier, and cyclophilin D [Halestrap et al., 2004]. Calcium overload and ROS induces opening of the MPTP which changes the membrane potential across the mitochondrial inner membrane, consequently resulting in mitochondrial swelling and subsequent apoptosis [Saitome et al., 2009].

It is reported that IPC prevents opening of the MPTP during ischemia /reperfusion downstream of GSK-3 $\beta$  [Juhaszova et al., 2004] or PKC- $\epsilon$  [Baines et al., 2003]. Glycogen synthase- 3 $\beta$  (GSK-3 $\beta$ ) signaling is also implicated in the regulation of cardiac hypertrophy and ventricular remodeling. Two isoforms (GSK-3A and GSK-3 $\beta$ ) exist and are equally expressed in cardiomyocytes, but it is the  $\beta$  isoform which is considered important in pathological hypertrophy [Matsuda et al., 2008]. The role of GSK-3 $\beta$  in cardiomyocyte hypertrophy has been thoroughly studied [Dorn et al., 2005]. GSK-3 $\beta$  is active in unstimulated cells. After stimulation it can be inhibited by two different mechanisms. One mechanism involves activation by growth factors and hypertrophic stimuli, which lead to the phosphorylation of a specific residue, the N-terminal serine 9 on GSK-3 $\beta$ . Unlike



most protein kinases, phosphorylation leads to inhibition of GSK-3 $\beta$ . Several kinases are capable of serine 9-induced phosphorylation and inhibition of GSK-3 $\beta$ , including p70S6 kinase, protein kinase A, protein kinase B and the protein kinase C family. Protein kinase B/Akt is the primary kinase responsible for phosphorylation and inhibition of GSK-3 $\beta$  after stimulation of cardiomyocytes with pro-hypertrophic agonists such as isoproterenol, phenylephrine and endothelin-1 [Goode et al., 1992; Cross et al., 1995]. The Wnt pathway is also implicated in the inhibition of GSK-3 $\beta$ . This pathway does not use serine-9 phosphorylation, but involves proteins called dishevelled and axin. This mechanism appears to sequester GSK-3 $\beta$ , limiting its access to the key downstream effector of the Wnt pathway, B-catenin.

#### **1.5.6.) Heat shock protein-70 (HSP-70)**

The cytoplasm of mammalian cells contain several families of molecular chaperones, including HSP-90, HSP-70, chaperone containing TCP1 (CCT, also called TCP1 – ring complex TRIC) and the small HSP (sHSP) family of proteins. The latter was **discussed in section 1.5.2**. The HSP-70 chaperone family consists of six member proteins. They are located in the cytosol and include HSP-70 and its cognate, HSc-70 [Daugaard et al., 2007]. HSP-70 proteins are highly abundant in the absence of stress, but are also inducible with stress. They are functionally highly homologous and are able to recognize hydrophobic surfaces of unfolded proteins and also partially folded intermediates. Their activity is controlled by their ability to bind ATP [Mayer et al., 2005]. HSP-70 proteins inhibit protein aggregation, thereby promoting productive folding of proteins [Young et al., 2010].

Various studies indicate that HSP-70 is protective against ischaemia/reperfusion injury. Expression of HSP-70 in rabbit hearts resulted in a reduction of ischaemia/reperfusion injury [Okubo et al., 2001]. Results from at least four studies demonstrated that transgenic overexpression of HSP-70 in mice hearts significantly protected against ischaemia/reperfusion injury [Marber et al., 1995; Plumie et al., 1995; Trost et al., 1998].

Many of the HSP's are regulated by the HSF family of transcription factors. HSF-1 expression can upregulate HSP expression and thereby protect against ischaemia/reperfusion injury [Zou et al., 2003]. Cardiac HSF-1 can be induced by transgenic overexpression of calcium/calmodulin-dependent kinase II (CAMK II), which is important in protecting the heart against ischaemia/reperfusion damage. CAMK II regulates calcium handling and regulates cell death in response to ischaemia/reperfusion injury. Expression of CAMK II protects against oxidative stress, hypoxia and angiotensin II induced apoptosis [Peng et al., 2010]. This cardioprotection is a result of increasing HSP-70 through phosphorylation of HSF-1. These studies suggest that HSF-1 may be a common regulator by which the cell induces a number of HSP's to protect against ischaemia/reperfusion injury.

At least 13 chaperones and co-chaperones assist HSP's with protein folding and/or targeting damaged proteins for degradation by the ubiquitin proteasome system in a process called protein triage [Arndt et al., 2007]. Increased expression of co-chaperones in the heart is associated with cardioprotection during ischaemia. A number of co-chaperones have been identified in the heart which control the activity of the chaperones, namely Bcl2-associated athanogene 1 protein (BAG-1) [Heymann, 2006], the carboxyl-terminus of HSP70 interacting protein (CHIP) [Zhang et al., 2005], the HSP-70/HSP-90 organizing protein (HOP) [Heymann, 2006] and the Heat Shock Protein- 40 family of proteins (DnaJ) [Qiu et al., 2006]. Studies have demonstrated that BAG-1 has the ability to inhibit apoptosis and to induce autophagy (discussed in section 2.1.) to protect myocytes. BAG-1 isoforms (BAG-1s and BAG-1L) are rapidly induced after ischaemia with the increase in BAG-1 being sustained after reperfusion [Towsend et al., 2004]. The interaction of BAG-1 with HSP-70 increases after ischaemia/reperfusion injury. Increasing BAG-1s and BAG-1L in cardiomyocytes reduce apoptosis after ischaemia/reperfusion injury. These studies demonstrated a cardioprotective role for BAG-1 with a critical component related to its interaction with HSC70/HSP-70. It has been demonstrated that autophagy plays an important role in the adaptation to ischaemia/reperfusion injury in association with BAG-1 [Gurusamy et al., 2009]. BAG-1 associates with the autophagosomal membrane protein LC3-II and may induce autophagy using HSc-70 [Gurusamy et

al., 2009]. BAG family members may shuttle damaged or oxidized proteins into the autophagy pathway to improve cell survival.

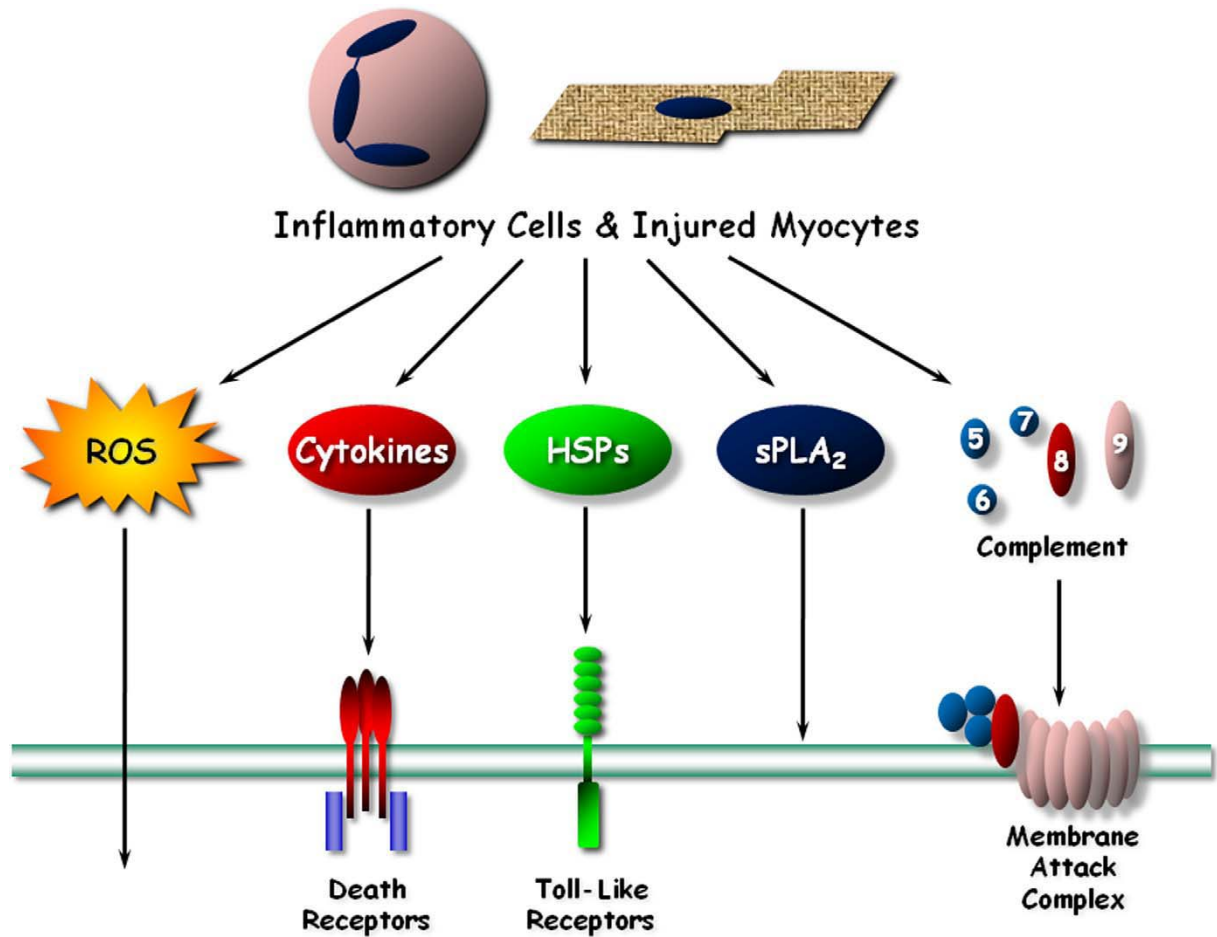
CHIP acts as a co-chaperone regulating HSP-70 in the folding system and is also important in the ubiquitin-proteasome system as an ubiquitin ligase. It has the ability to ubiquitinate proteins that the chaperone/co-chaperone complex is unable to refold. CHIP plays an important role in the heart in ischaemia/reperfusion injury [Zhang et al., 2005] and has a critical role in shuttling damaged and oxidized proteins into the autophagic pathway after ischaemia/reperfusion. CHIP increases in the heart in response to high glucose levels and is responsible for the degradation of the pro-hypertrophic transcription factor, GATA4 [Kobayashi et al., 2007]. Chaperones and co-chaperones are thus important regulators of protein quality control in the heart.

## 1.6) Modalities of Post- Reperfusion Cell Death

Three major forms of cell death exist in the myocardium: **apoptosis**, **necrosis** and **autophagy**. The majority of cardiomyocyte death (adding to the final infarct) occurs during ischaemia and the first few minutes of reperfusion [Zhao et al., 2001]. Unfortunately, cell death does not stop there and can continue in the post-ischaemic zone from days to even weeks, post-reperfusion [Zhao et al., 2000].

All three modalities of cell death may contribute to the continued loss of cardiomyocytes in the post-ischaemic zone or the reperfused area. The following mechanisms can contribute to the continuous myocyte death that makes up the final infarct: (i) myocytes which have been irreversibly damaged despite reperfusion; (ii) continued dysfunction of the coronary microvasculature; and (iii) the infiltration of inflammatory cells [Baines et al., 2011]. These processes (shown in **Figure 1.3**) can lead to myocyte death as a result of elevated myocardial concentrations of ROS [Zweier et al., 2006], activation of Toll-like receptors [Kim et al., 2009; Chao et al., 2009], secretion of toxic cytokines [Vinten-Johansen et al., 2004; Zang et al., 2006] and activation of the complement cascade [Vinten-Johansen et al., 2004; Frangogiannis et al., 2002].

The end result of myocardial ischaemia is energy stress. Reperfusion however, is accompanied by sudden ionic shifts and substantial oxidative stress. After acute ischaemia/reperfusion injury the myocardium is subjected to biomechanical stress and inflammation. Some cells die by apoptotic- and necrotic pathways, while the surviving cells are subjected to metabolic and functional changes [Gottlieb et al. 2011]. The **goal** of cardioprotection is to prevent cell death during acute ischaemia/reperfusion injury and to modulate metabolic and functional changes after the injury to sustain healthy mitochondrial function.



**Figure 1.3:** Mediators of post-reperfusion cardiomyocyte death. A variety of humoral and cellular factors are released from dying cardiac myocytes and inflammatory cells during reperfusion that are cytotoxic. The 5 main factors are:

- reactive oxygen species (ROS);
- inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ );
- heat shock proteins (HSPs) which activate Toll-like receptors;
- secretion/release of enzymes such as secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>) which directly attacks myocyte membranes; and
- activation of the complement cascade leading to the formation of the cytolytic membrane attack complex [Baines et al., 2011]

The functional status of cardiomyocytes is dependent on mitochondria, capable of producing ATP, which is essential for the energy needs and calcium homeostasis of the cell. The cardiac mitochondrion acts as a global signal integrator of cellular homeostasis and is the key determinant of cardiomyocyte fate, as shown in **Figure 1.4**. [Gottlieb et al., 2011]. Poorly functional mitochondria can determine the fate of the cell through activation of either apoptotic or necrotic cell death [Gottlieb et al., 2011].

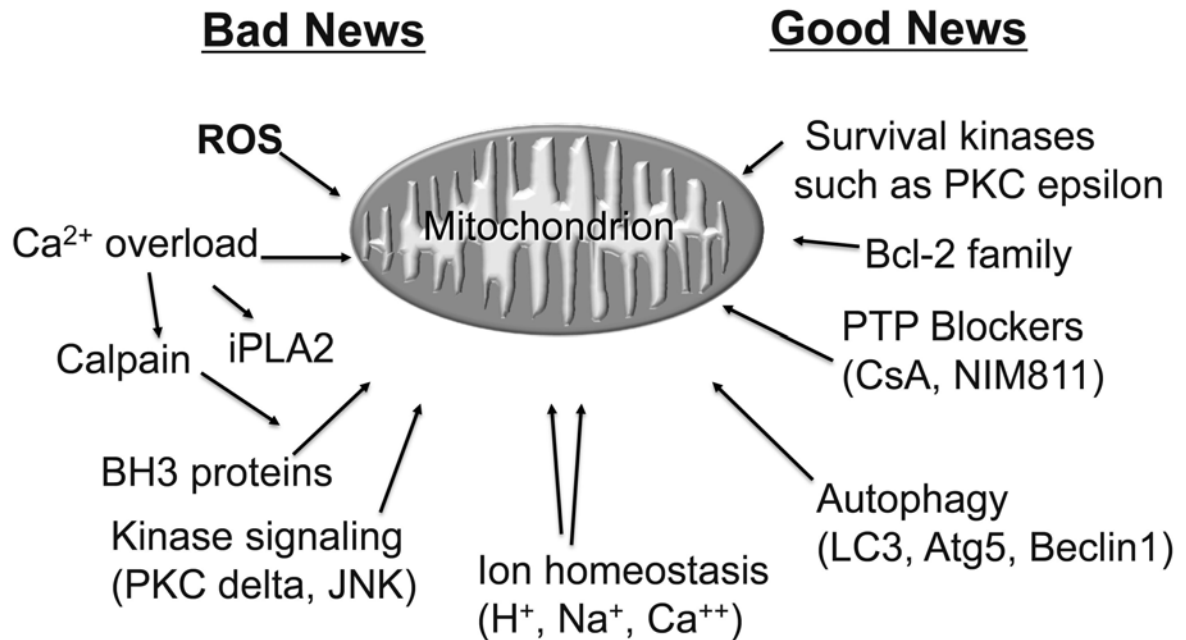
The signals that destabilize mitochondria include reactive oxygen species (ROS), PKC- $\delta$ , JNK and pro-apoptotic members of the Bcl-2 family (Bax/Bak and a BH3-only family member such as Bid, Bnip3, Nix) [Logue et al., 2005; Gustafsson et al., 2006]. The combined effect of calcium overload and ROS production can lower the threshold for the activation of mitochondrial iPLA<sub>2</sub>, resulting in the production of arachidonic acid and plasmalogens which are detrimental to mitochondria [Ford et al., 1991]. In contrast, signals that promote stabilization of mitochondrial function, as summarized in **Figure 1.4**, include PKB/Akt and its downstream targets, PKC- $\epsilon$  and the pro-survival members of the Bcl-2 family [Gustafsson et al., 2006].

### 1.6.1.) Apoptosis

One of the most widely recognized biochemical features of apoptosis is the activation of a class of cysteine proteases known as **caspases** [Thornberry & Lazebnik, 1998]. Caspases are present in the cell as inactive procaspases that are cleaved and activated in response to apoptotic stimuli. Initial activation of caspases may involve transduction of a signal from **membrane death receptors** belonging to the tumor necrosis factor receptor (TNF) family, such as Fas and TNF receptor 1 (TNFR1) [Ashkenazi & Dixit, 1998], or may be mediated by a **mitochondrial pathway** [Green & Reed, 1998].

Stimulation of death receptors results in the activation of caspase-8, which goes on to activate caspase-3, a key effector protein of the apoptotic machinery. Release of cytochrome c from the mitochondria, on the other hand, leads to activation of caspase-9 through formation of a cytosolic complex and subsequent activation of caspase-3.

Unlike necrosis, apoptosis is energy dependent and highly regulated. Apoptosis is controlled by the complex interaction of numerous prosurvival and prodeath signals. These include the **Bcl-2 family of proteins**, which may be antiapoptotic (Bcl-2, Bcl-X<sub>L</sub>) or proapoptotic (Bad, Bid), and exerts its effects primarily at the level of mitochondria [Adams & Cory, 1998]. Other important regulators of apoptosis act at the level of caspases. Such proteins include **cellular FADD-like inhibitory protein (cFLIP)** and the **inhibitor of apoptosis (IAP) family** [Rasper et al., 1998; Uren et al., 1998]. There are also proteins that counteract the effect of the caspase inhibitors themselves such as Smac/DIABLO, a mitochondrial protein that, when released, binds and neutralizes IAPs, thus promoting caspase activity [Srinivasula et al., 2000]. Several other factors are also believed to be involved in the regulation of apoptosis including, growth factors, **mitogen activated protein kinases (MAPKs)**, **PKB/Akt**, calcium, and oxidants. The complex interaction of all these molecules determines the ultimate fate of the cell: life or death.



**Figure 1.4:** Mitochondria integrate the signals of ischaemia/reperfusion injury. Mitochondria respond to a variety of inputs, both good and bad, including ROS, phospholipases, kinases, phosphatases, Bcl-2 family members (both pro- and anti-apoptotic), calcium, and the pH of the cytosol and mitochondrial matrix. See text for details [Gottlieb, 2011].



### 1.6.1.1 Caspases, the proteases of the apoptotic pathway

As stated before, a key phenomenon of apoptotic cell death is the activation of a unique class of aspartate-specific proteases known as caspases. All caspases are composed of a prodomain and an enzymatic region. Heterogeneity among the proteases exists regarding the structure of the prodomain, suggesting that this region may define important functional differences between caspases. Caspase-1, -2, -4, -5, -8, -9, and -10 contain a long prodomain of  $\approx 15$  to 25 kDA compared with  $<5$  kDA in caspases-3, -6, and -7. For activation, the caspase proform has to be cleaved into a large subunit and a small subunit within the enzymatic domain that finally reassociate to form a complex comprising two small and two large subunits. The prodomain is not necessary for the proteolytic activity once the caspase is activated. Interestingly, all activating cleavages occur after an aspartate residue. Since this cleavage site is a unique characteristic of caspases (with the serine protease, granzyme B, being the only exception), activation can occur only through auto activation or cleavage by another caspases or granzyme B.

Cells possess multiple caspases, which may work in a cascade fashion. The redundancy may serve to amplify and accelerate the response, as well as to provide multiple mechanisms to complete the process. Indeed, the redundancy is apparent in the phenotypes of knockout mice in which the deletion of a single caspase is associated with a relatively unimpressive phenotype. Deletion of caspase-1 or caspase-11 does not result in dramatic phenotypic changes; thus the roles for these caspases are less clear [Li et al., 1997; Wang et al., 1998]. However, deletion of caspase-3 results in a failure of neuronal apoptosis, and the mice are born with abnormally large brains and die soon after birth [Kuida et al., 1996].

Substrates for caspases comprise many different proteins, including nuclear proteins, proteins involved in signal transduction, and cytoskeletal targets [Cardone et al., 1997; Kothakota et al., 1997; Sakahira et al., 1998]. Most of these protein substrates appear to be cleaved by caspases-3 and -7.

In summary, caspases can be grouped into an upstream (caspases -1, -2, -4, -5, -8, -9, -10) and downstream (caspases -3, -6, -7) subgroup. Upstream caspases are characterized by long prodomains that appear to contain essential regulatory proteins. Most of the activity that finally leads to the lethal proteolytic breakdown of cellular target proteins is exerted by downstream caspases sensitive to DEVD (aspartate-glutamine-valine-aspartate) oligopeptides (caspase-3 and caspase-7).

#### **1.6.1.2 Mechanisms of caspase activation**

Once downstream caspases are activated, cell death appears to be inevitable. Therefore, understanding the mechanisms that initiate proteolytic activation of these caspases is a crucial step in defining targets that allow for the modulation of apoptotic cell death. Recent data suggest that activation of caspases may take place either within **death receptor complexes** of the cytoplasmic membrane or by a **mitochondrion-dependent mechanism** within the cytosol.

##### **1.6.1.2 (a) Death- and decoy receptor pathway**

The best characterized pathways for the initiation of apoptosis involves the binding of extracellular death signal ligands (FasL and TNF) to receptors that belong to the TNFR gene superfamily [Smith et al., 1994]. The cytoplasmic sequence divides the TNFR superfamily into two main subgroups of receptors that either possess or lack a 'death domain' (DD) [Tartaglia et al., 1993; Itoh & Nagata, 1993]. The death-domain-containing receptors, or 'death receptors' (DR) include TNFR1, Fas (Apo1/CD95), DR3 (Apo3), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and DR6. The death receptors interact via their DD with intracellular DD-containing adaptors, such as FADD (Fas-associating protein with DD) and TRADD (TNF receptor-associated death domain), and recruit these adaptors to the cell membrane. Thus, binding of Fas ligand to the Fas receptor leads to clustering of the Fas receptor's DD. The adapter, FADD, then binds through its own DD to the cluster receptor DD. FADD also contains a 'death effector domain' (DED) that binds to an analogous domain within the pro-caspase-8 protein. Upon recruitment by FADD, pro-caspase-8 oligomerization drives its own activation (to caspase-8) through self-cleavage. Caspase-8 then activates downstream effector caspases, such as caspase-3, thereby initiating apoptosis. Signaling induced by

activation of TNFR1 or DR3 diverges at the level of TRADD [Hsu et al., 1995]. On the one hand, nuclear translocation of the transcription factor nuclear factor  $\kappa$ B (NF $\kappa$ B) and activation of c-Jun N-terminal kinase (JNK) are initiated [Chinnaiyan et al., 1996; Hsu et al., 1996; Yeh et al., 1997]. On the other hand, TNF- $\alpha$  signaling is linked to Fas signaling pathways through interaction of TRADD with FADD. Surprisingly, FADD knockout mice exhibit a phenotype of ventricular thinning and poorly developed trabeculation of the heart [Yeh et al., 1998]. An additional death domain-containing protein, receptor-interaction protein (RIP), was also shown to interact with the cytoplasmic domain of TNFR1 [Stanger et al., 1995; Hsu et al., 1996]. Caspase-2 can be recruited to RIP through an adaptor protein called RIP-associated ICH/Ced-3-homologous death domain protein (RAIDD) [Duan et al., 1997].

Another subgroup of TNFR-homologues consists of decoy receptors, which function as inhibitors, rather than transducers of signaling. This subgroup includes decoy receptor (DcR)1 and DcR2 (both of which are cell-surface molecules) as well as osteoprotegerin (OPG) and DcR3 (both of which are secretory, soluble proteins).

#### **1.6.1.2 (b) Mitochondrial pathway**

The other well-characterized pathway to caspase activation involves participation of the mitochondria. In 1997, Zou and co-workers showed that cytochrome c participates in caspase activation, in concert with other proteins that were subsequently identified as caspase-9 and Apaf-1. Apaf-1 contains a region shared with other caspases and may be required for protein-protein interaction. In addition, it has a binding site for ATP (or dATP), and a series of 12 WD-40 repeats, which may be involved in binding cytochrome c [Zou et al., 1997]. Apaf-1 interacts with cytochrome c and dATP to activate caspase-9 [Li et al., 1997]. The activated and processed caspase-9 then cleaves caspase-3 to generate the active enzyme, which is the effector protease that proceeds to degrade most of the cellular targets.

This pathway requires cytochrome c to be released from the mitochondria, where it is normally sequestered in the intermembrane space. Thus, agents affecting

mitochondrial integrity may lead to cytochrome c release and activation of caspases. Little information is available explaining how cytochrome c release is controlled, or even if this is a nonspecific event accompanying general loss of mitochondrial integrity or a regulated release of a single protein. It appears to occur before loss of mitochondrial membrane potential [Kluck et al., 1997], but may represent generalized leakage of intermembrane space constituents. Adachi and co-workers (1998) have shown that loss of outer membrane integrity is a rather late event, but far earlier, cytochrome c has become unavailable for electron transport, although still present in the intermembrane space. This suggests its interaction with other protein(s) that eventually may all be released from the mitochondria when the outer membrane ruptures. One postulated mechanism for the rupture of the outer mitochondrial membrane to occur is swelling of the matrix [Vander Heiden et al., 1997]. Since the inner membrane is heavily folded, it can tolerate swelling in response to ion fluxes without rupture, however, as it swells, it will stretch and eventually rupture the outer membrane. Disruption of mitochondrial calcium homeostasis, leading to the formation of the permeability transition pore, may also result in mitochondrial matrix swelling and rupture [Crompton, 1999].

A widely recognized target of caspases is the enzyme poly-(ADP-ribose) polymerase (PARP). PARP was the first cellular protein to be identified as being specifically cleaved in apoptosis [Kaufmann, 1989], and its cleavage subsequently was shown to be a universal phenomenon occurring in apoptosis induced by a variety of stimuli [Kaufmann et al., 1993]. Specific proteolysis of PARP occurs in the DNA binding domain and as a result, two polypeptides of 24 and 89 kDa are formed, thereby abolishing PARP's catalytic activity [Nicholson et al., 1995; Tewari et al., 1995]. An apparent active role for PARP in apoptosis came from studies using human osteosarcoma cells that undergo spontaneous apoptosis. Smulson and co-workers (1997) observed transient PARP activation in the early phase of apoptosis, prior to caspase-mediated cleavage and inactivation of PARP protein. This transient burst of poly-(ADP-ribosyl)ation of nuclear proteins was subsequently detected at an early stage of induced apoptosis in other cell types [Simbulan-Rosenthal, 1998]. These findings suggest that PARP and poly-(ADP-ribosyl)ation may trigger an important step of early apoptosis. However, treatment of PARP<sup>-/-</sup> fibroblasts with TNF- $\alpha$ , anti-Fas and IL-

3 withdrawal [Menissier-de Murcia et al., 1997; Wang et al., 1997], and of thymocytes with  $\gamma$ -irradiation, dexamethasone, ceramide and etoposide induces normal apoptotic responses [Leist et al., 1997; Wang et al., 1997]. Moreover, PARP<sup>-/-</sup> primary neurons and hepatocytes undergo apoptosis normally in response to a variety of stimuli, including potassium withdrawal, staurosporine, and colchicine [Leist et al., 1997]. These results indicate that at least in certain cell types, PARP does not actively participate in the apoptotic cascade. Despite the above-mentioned discrepancies, most studies point out that PARP is specifically cleaved during apoptosis, and that massive DNA damage activates PARP, before or in parallel to caspase activation, leading to necrosis. Therefore, it remains to be elucidated whether the activation and/or cleavage of PARP has a specific role in the “decision-making” phase or activating phase of programmed cell death.

An important question, which is often raised, is why must PARP be cleaved in cells destined to die? It has been postulated that PARP cleavage occurs in order to prevent depletion of energy pools required for later stages of apoptosis [Earnshaw, 1995]. Transient transfection of cells with non-cleavable PARP delays apoptosis induced by anti-Fas, as judged by morphological criteria such as cell shrinkage and nuclear condensation. The authors claimed that PARP cleavage facilitates cellular disassembly ensuring cell death completion [Olivier, 1998]. However, in two other studies, cell lines stably expressing non-cleavable PARP exhibit increased apoptosis in response to TNF- $\alpha$  and staurosporine, as determined by extensive morphological analyses [Herceg & Wang, 1999; Boulares et al., 1999]. It was hypothesized that mutant PARP accelerates apoptosis through compromised energy metabolism and the mitochondrial permeability transition (MPT) [Nicholson & Thornberry, 1997], or by shifting caspase-3 activation to an earlier time point [Boulares et al., 1999]. Alternatively, poly(ADP-ribosyl)ation of histones and other nuclear proteins by non-cleavable PARP could also substantially contribute to unwinding of chromatin structures thereby facilitating access to apoptotic DNAase(s). The apparent discrepancy of the results between the above-mentioned studies using non-cleavable PARP can be attributed to the technical means by which the mutant PARP was introduced into the target cells, i.e. transient versus stable transfection. More importantly, the observed induction of massive necrosis coupled with NAD<sup>+</sup> and ATP depletion in

mutant PARP expressing cells [Herceg & Wang, 1999], strongly argues that PARP cleavage plays an important role in ensuring the normal speed and order of apoptotic events, and prevention from necrosis in cells undergoing apoptosis. Since massive activation of PARP leads to necrotic cell death, cells that die via apoptosis must block PARP-mediated necrosis by cleaving PARP. Together, these findings fit well with the proposal that PARP cleavage has a function in the prevention of necrotic death that would otherwise lead to pathological inflammatory responses [Earnshaw, 1998]. This theory is also supported by several other studies demonstrating that PARP activation is involved in inflammatory responses [Szabo et al., 1997, 1998; Cuzzocrea et al., 1999; Olivier et al., 1999]. Moreover, streptozotocin-induced disruption of pancreatic  $\beta$ -cells underlines PARP activation and energy depletion as a cause of necrotic cell death in this cell type [Burkart et al., 1999].

#### **1.6.1.3 Regulatory proteins**

In apoptosis, protein-protein interactions are the underlying theme in both mitochondria and death receptor pathways. A sophisticated and tightly controlled network of protein-protein interactions exists to ensure the accuracy of the cell-death machinery.

The **Bcl-2 family** is a large key group of apoptosis regulators which, through the diverse interactions among themselves and with other proteins, control the release of apoptogenic factors needed for caspase activation [Adams & Cory, 1998; Chao & Korsmeyer, 1998]. The **IAP (Inhibitor of apoptosis protein)** family is another family of proteins that inhibits apoptosis through physically interacting with caspases and thereby directly inhibiting their function.

##### **1.6.1.3 (a) Bcl-2 Protein Family**

Members of the Bcl-2 family include both anti-apoptotic proteins, exemplified by Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, Bcl-w and A-1, and pro-apoptotic proteins exemplified by Bax, Bak, Bik, Bad and Bid. In terms of sequence, Bcl-2 family proteins share at least one of four homologous regions termed Bcl homology (BH1 to BH4). Based on sequence homology, a subclass of pro-apoptotic proteins termed “BH3-only”

can be classified that share sequence homology only in the BH3 domain. While all of the pro-apoptotic members use the BH3 domain to interact with anti-apoptotic proteins, BH3-only proteins, including Bad and Bid, appear to act mainly as antagonists of anti-apoptotic members such as Bcl-2 and Bcl-X<sub>L</sub>. In contrast to the opposing biological functions and wide differences in amino-acid sequences, experimentally determined crystal structures of Bcl-2 [Petros et al., 2001] and Bcl-X<sub>L</sub> [Muchmore et al., 1996; Aritomi et al., 1997], vs Bax [Suzuki et al., 2000] and Bid [McDonnell et al., 1999; Chou et al., 1999] are surprisingly similar.

The mechanism by which Bcl-2 family proteins regulate apoptosis has been a subject of intensive research. Currently it remains controversial and several models have been proposed. An attractive mode of action is the heterotrimerization between anti-apoptotic and pro-apoptotic Bcl-2 family members [Oltvai et al., 1993; Yang et al., 1995; Reed et al., 1996]. Some information about the structural basis of these interactions is provided by the three-dimensional structure of Bcl-X<sub>L</sub> in complex with a peptide derived from the BH3 domain of Bak [Sattler et al., 1997]. The structure reveals a hydrophobic surface pocket on Bcl-X<sub>L</sub> formed by the BH1-3 domains bound by the Bak BH3 domain peptide in helical conformation. Since the BH3 domain is buried in the structure of pro-apoptotic proteins Bid [McDonnell et al., 1999; Chow et al., 1999] and Bax [Suzuki et al., 2000], this raises the speculation that conformational changes are necessary for the exposure of the BH3 domain of a pro-apoptotic protein and its inhibition of the functional pocket on the anti-apoptotic partner. In the cell environment, pro-apoptotic Bcl-2 family members are suggested to undergo such conformational changes [Desagher et al., 1999] triggered by dephosphorylation [Zha et al., 1996] or proteolytic cleavage by caspases (such as the cleavage of Bid to generate tBid [Slee et al., 2000; Li et al., 1998; Luo et al., 1998]).

#### **1.6.1.3 (b) IAP (Inhibitor of apoptosis protein) family**

The Bcl-2 and IAP families are regulators of caspases at two different levels: the Bcl-2 family controls signaling events upstream of caspases, while the IAP family directly binds and inhibits caspases. The human IAP family contains eight distinct



cellular members including X-IAP (X-linked IAP), c-IAP1, c-IAP2, and survivin [Devereaux et al., 1999]. In humans, IAPs such as X-IAP, c-IAP1, and c-IAP2 selectively inhibit caspase-3, -7 and -9 through direct molecular interactions but not caspase-1, -6, -8, -10. In addition, IAPs can interact with Smac/DIABLO, which is released from the mitochondria together with cytochrome c upon death stimuli. The binding of Smac/DIABLO removes IAPs from their association with caspases and thus relieves their caspase-inhibiting function [Devereaux et al., 1997; Roy et al., 1997].

In the human heart, cardiac failure is characterized by the progressive death of myocytes [Olivetti et al., 1997]. It was always believed that all adult cardiomyocyte cell death was caused by necrosis. However, Narula et al. [1996] and Olivetti et al. [1997] provided the first evidence that apoptosis occurs in the myocardium of patients with end-stage dilated cardiomyopathy. Similarly, Saraste et al. [1997] demonstrated the presence of apoptotic cells in the infarct and peri-infarct regions of myocardium obtained from patients dying from a recent myocardial infarction. Probably the best information about apoptosis in the heart has been derived from studies of experimental ischaemia/reperfusion. Kajstura and co-workers [1996], have shown that apoptosis is the predominant mode of cell death in ischaemia-reperfused tissue.

Since the significance of cell death by apoptosis to cardiac pathology has been established, there is still great interest in the field. This is because unlike necrosis, which is thought to be an essentially irreversible process, the step-by-step nature of apoptosis suggests it may be amenable to therapeutic intervention.

### **1.6.2.) Autophagy**

Autophagy is a catabolic process which degrades long-lived proteins and organelles, thereby maintaining cardiac structure and function and inhibiting cardiac abnormalities. It is an evolutionary conserved lysosomal pathway, involving the degradation of damaged cytoplasmic proteins, macromolecules and organelles such as ribosomes, the endoplasmic reticulum, Golgi-apparatus and mitochondria. The term autophagy is derived from the Greek language “auto”



(self) and phagy (to eat) and means self-digestion. It is a highly regulated, physiological process, initiated by starvation (amino acid and nutrient deficiency), hypoxia and metabolic stress. Self-digestion provides a means of recycling extracted amino acids and fatty acids for ATP production via the TCA cycle and thereby supplying an alternative energy source which may serve as a temporary survival mechanism.

It is therefore a protective cell survival mechanism against environmental and cellular stress. However, if the cellular stress leads to continuous or excessively induced autophagy, cell death may occur. This type of cell death is referred to as type II programmed cell death (CPD-type II) [Mizushima et al., 2008]. Autophagic cell death is caspase-independent and does not involve classic DNA laddering [Kirisako et al., 1999].

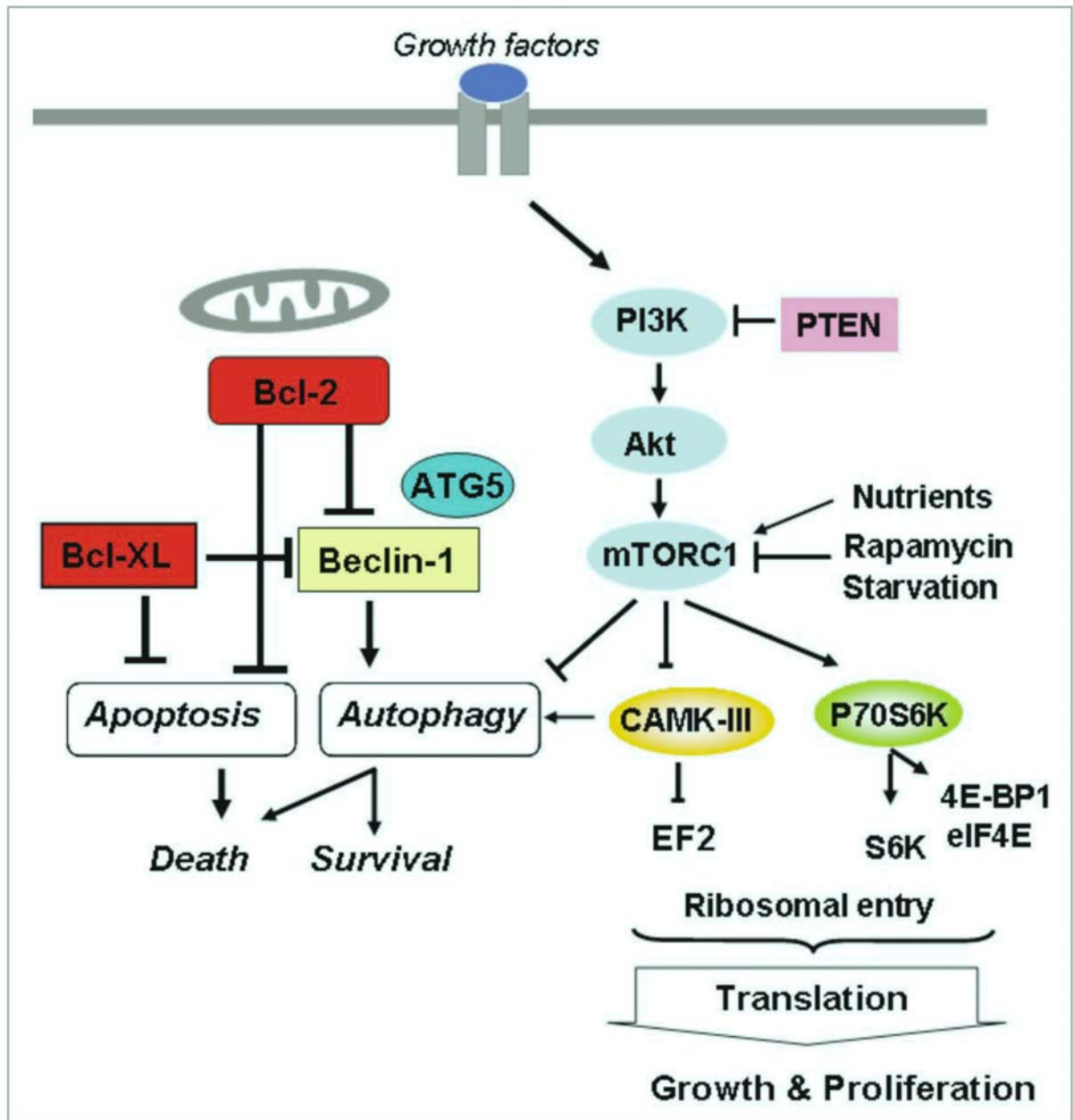
The process of autophagy consists of four stages; induction, nucleation, expansion and maturation/retrieval of autophagosomes [He et al., 2009; Levine et al., 2004]. **Induction and nucleation** involves the formation of an isolation membrane known as a phagophore from the source which can be the endoplasmic reticulum or mitochondria [Axe et al., 2008]. Phagophores migrate to the phagophore assembly site (PAS), where the factors involved in the autophagic process are recruited. Phagophores undergo expansion at the PAS site, after which they engulf the damaged cytosolic components (cargo), and subsequently develop into mature autophagosomes. Mature autophagosomes bind to microtubules and are transported to lysosomes for fusion. Autophagosome formation is regulated by the autophagy-related genes (ATG). Phagophore induction and expansion are mostly triggered by the class-III phosphoinositide 3-kinase, Vps34, which forms a multi-protein complex with Beclin-1, Atg14 and Vps150, leading to the formation of phosphatidylinositol 3-phosphate (PtdIns3P). The latter is necessary for recruitment of other regulatory proteins at the phagophore assembly site to facilitate the expansion of the phagophore. Phagophore formation is also induced by a macromolecular complex formed by Atg13, Unc-51-like kinase 1 (Ulk 1, a homologue of the yeast Atg1) and Ulk 2, which phosphorylates the focal adhesion kinase family interacting protein 200 (FIP200), which triggers initiation of autophagy.

**The elongation phase** involves two parallel ubiquitin ligase-like systems. Firstly, Atg12 must be conjugated onto Atg5 in a reaction catalyzed by Atg7 and Atg10. This is a pre-requisite step for the subsequent conjugation of LC3 onto phosphatidylethanolamine in the growing autophagosomal membrane [Gustafsson et al., 2008]. These two systems act in parallel and are well coordinated, thus enabling the formation of autophagosomes which are recruited to engulf targets through the action of an adaptor protein, such as p62. The Atg12-Atg5 complex binds to the phagophore membrane and promotes its elongation. Subsequently, Atg7, Atg3 and the Atg12-Atg5 complex modulate the conjugation of the lipidated form of LC3 known as LC3-II. LC3-II is crucial for autophagosome expansion, including membrane tethering and hemifusion as well as controlling the size of the autophagosome by regulating the membrane curvature. Once autophagosomal formation is accomplished, LC3-II is released into the cytosol after its Atg4-mediated cleavage from phosphatidylethanolamine. The mature autophagosome transports its cargo to the lysosome for degradation. The degraded products which include amino acids, carbohydrates, nucleic acids and fatty acids are eventually exported by the lysosome.

Studies demonstrated that autophagy can be induced by cytotoxic signals in cells that are resistant to apoptosis, such as those lacking Bax or Bad or those expressing high levels of Bcl-2 or Bcl-X<sub>L</sub> [Shimizu et al., 2004]. Furthermore, apoptosis and autophagy are intertwined (shown in **Figure 1.5**) and linked by the following effector proteins; Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, ATG5 and p53 [Shimizu et al., 2004; Pattingre et al., 2005; Yosefi et al., 2006; Lockshin et al., 2007; Akar et al., 2008]. Apoptosis and autophagy share some common signal transduction pathways, such as PI3-K/AKT/mTOR, NF $\kappa$ B, and ERK p44/p42 MAPK pathways [Dalby 2010].

Despite the enormous advances in the elucidation of the mechanism of autophagy, the signal transduction pathways that regulate autophagy are still not clear [Xie et al., 2007]. Mechanistic evidence exists however, which links the kinases to known regulators of autophagy (**Figure 1.6**). AMPK has been shown to inhibit the phosphatidylinositol kinase homolog; mammalian target of rapamycin (mTOR) under conditions of energy stress, thereby induces a protective autophagic response [Papandreou et al., 2008]. mTOR is activated by

nutrients and amino acids and is an important negative regulator of autophagy [Meijer et al., 2004]. JNK has been shown to increase the expression of Beclin-1 in a c-Jun dependent way [Li et al., 2009]. Beclin-1 has an important role in autophagy where it is recruited to the protein complex containing the lipid kinase, Vps34, which triggers nucleation of autophagosomes [Liang et al., 2006; Takahashi et al., 2007]. JNK further promotes autophagy by inducing the release of Beclin-1 from an inhibitory Bcl-2-Beclin-1 complex [Wei et al., 2008 & 2009].

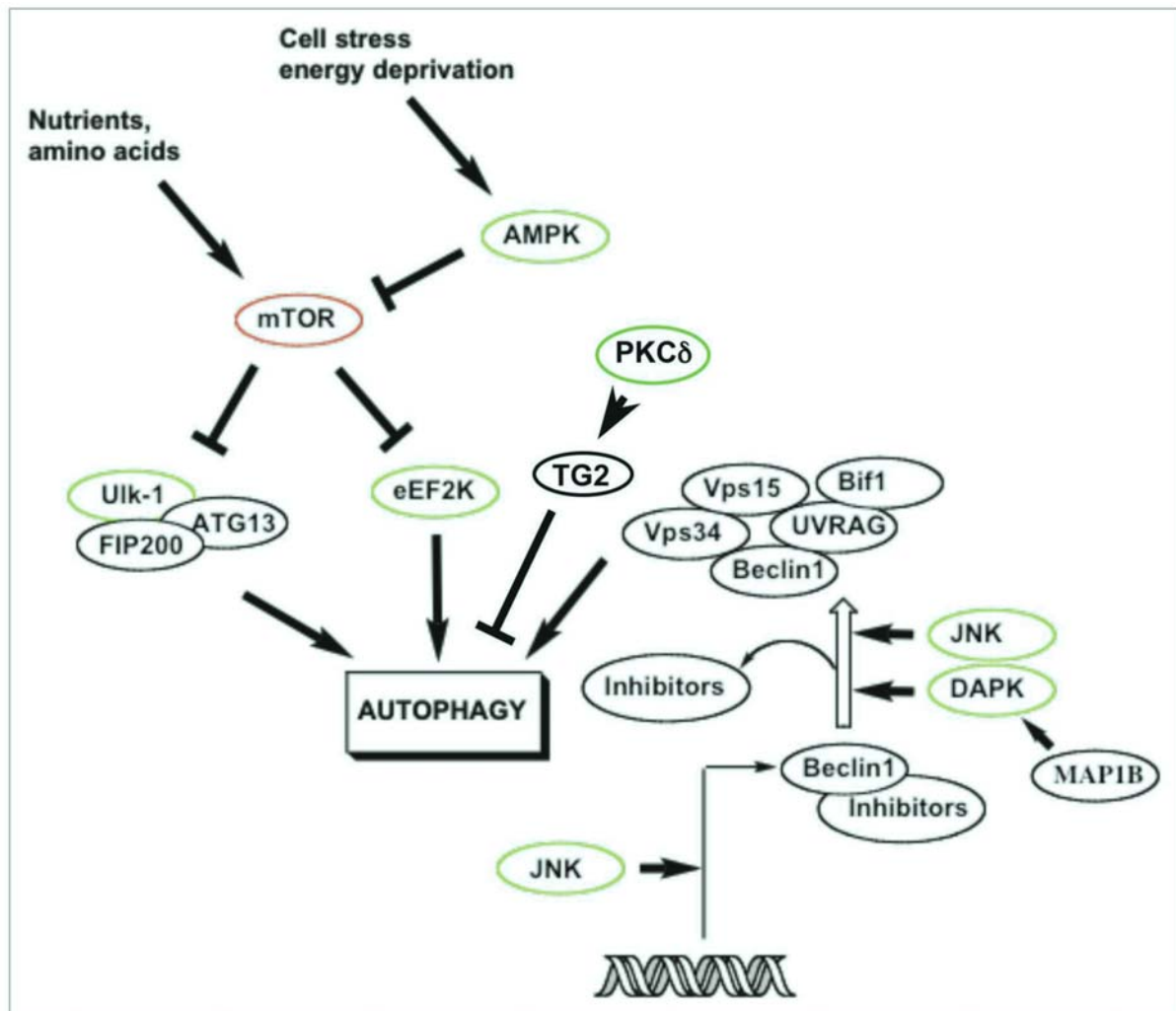





**Figure 1.5:** Regulation of autophagy and apoptosis is intertwined. Downstream targets of mTOR, EF2K (a.k.a.CAMK-III) and P70S6K, play a role in the regulation of translation and autophagy (see text for details) [Dalby, 2010].

The protein serine/threonine kinase, eEF2K, is negatively regulated by mTOR and promotes autophagy during periods of cell stress. These studies suggest a possible link between the inhibition of protein synthesis and the induction of autophagy [Hait et al., 2006; Wu et al., 2006 & 2009]. Autophagy can also be induced by PKC and NO but the mechanisms involved are currently unknown [Huang et al., 2010].

It has been recognized since 1980, that autophagy occurs in cardiac tissue [Decker et al., 1980]. Autophagy could be detected under basal conditions in the heart and deletion of Atg5 in adult mice hearts rapidly induced cardiac hypertrophy, left ventricular dilatation and contractile dysfunction. This was associated with an increase in protein ubiquitination and aggregation, disorganized sarcomere structure, endoplasmatic reticulum stress and mitochondrial dysfunction [Nakai et al., 2007]. Autophagy is also essential for maintaining normal cardiac function during aging. Deletion of Atg5 in the mouse heart during aging resulted in cardiac dysfunction and aging cardiomyopathy [Taneike et al., 2010]. These results demonstrated that a certain level of autophagy is a requirement for normal cardiac structure and function. A modest increase of autophagy under stress conditions appears to be protective. A massive activation however, may be detrimental to the heart [Nishida et al., 2009; Wang et al., 2011].

The initial studies, conducted in 1980, demonstrated that induction of autophagy correlated with functional recovery of the rabbit heart after ischaemia/reperfusion [Decker et al., 1980]. The same study demonstrated that prolonged ischaemia impair the autophagosome-lysosomal pathway, which is associated and correlated with contractile dysfunction and irreversible damage. Another study reported the expression of several autophagy-related genes during a period of prolonged ischaemia [Vatner et al., 2005]. Autophagy is stimulated during hypoxia and/or ischaemia in the heart, when the energy reserves is rapidly depleted [Nishida et al., 2009]. The decrease in cellular ATP content during ischaemia stimulates AMPK, which acts as an energy sensor for energy deprivation. Activation of AMPK during ischaemia is required for induction of protective autophagy, since inhibition of AMPK during sustained ischaemia significantly suppresses autophagy [Matsui et al. 2007].



**Figure 1.6:** Protein kinase signaling pathways implicated in the regulation of autophagy. JNK-1 activates autophagy by promoting the expression of the autophagy related genes (indicated by the light arrow). Phosphorylation of BCL-2 by JNK-1 disrupts the Beclin-1 BCL-2 complex and the inhibiting BCL-2 is released from the complex (darker arrow) and Beclin-1 is recruited to the Vps34 complex and autophagy is initiated (light block arrow, ). Inhibition of a particular kinase or kinases may help blocking induction of protective autophagy. AMPK is activated by nutrient deprivation (indicated by dark arrow ), and inhibits mTOR (indicated by dark arrow ), which negatively regulates autophagy. Inhibition of mTOR by AMPK induces protective autophagy. eEF2K is negatively regulated by mTOR. DAPK promotes autophagy and is activated by MAP1B. Inhibition of some kinases, such as PKCδ, may lead to autophagic cell death. See text for details [Dalby, 2010]

Inhibition of autophagy was accompanied by enlargement of the infarction [Takagi et al., 2007]. Further studies support these findings that activation of autophagy during ischaemia is generally protective and essential for cell survival and also for maintenance of cardiac function [Matsui et al., 2007].

The level of autophagy further increases during reperfusion and the production of ROS was suggested to be the main inducer of autophagy during reperfusion. Production of ROS was accompanied by a robust activation of the autophagic mediator, Beclin-1, during reperfusion [Hariharan et al., 2011]. In a different study, Beclin-1 was also upregulated during reperfusion and this was associated with increased autophagy as measured by LC3 puncta. Mice with reduced levels of Beclin-1 had smaller infarcts after 24 hours of reperfusion [Matsui et al., 2007; Hariharan et al., 2011]. These results suggest that enhanced autophagy during reperfusion is detrimental in the heart [Matsui et al., 2007]. It would appear that the “good” autophagy is AMPK dependent, while the “bad” autophagy is dependent on Beclin-1 [Matsui et al., 2007].

Subsequently, it was demonstrated by several laboratories that autophagy increased in the setting of IPC [Yitzhaki et al., 2009; Yan et al., 2009]. This involvement of autophagy with IPC has been suggested to serve a protective function [Gurusamy et al., 2009; Park et al., 2009]. A recent study also demonstrated that IPC induces autophagy [Huang et al., 2010]. Using the rat Langendorff perfusion model, the results indicated that the autophagy marker p62 was downregulated after the IPC protocol, compared to control hearts. Results from the same study demonstrated that autophagy is required for cardioprotection by IPC. Langendorff-perfused rat hearts were pretreated with an inhibitor of autophagy, 15 min before the IPC protocol. These hearts presented with larger infarcts than non-IPC hearts at the end of 2 hours of reperfusion. These results suggest that autophagy may be a possible end effector of IPC [Huang et al., 2010].

### **1.6.3.) Necrosis**

Mitochondria may also trigger cell death through another pathway, known as the mitochondrial permeability transition (MPT), first described in 1976 [Hunter et al.,

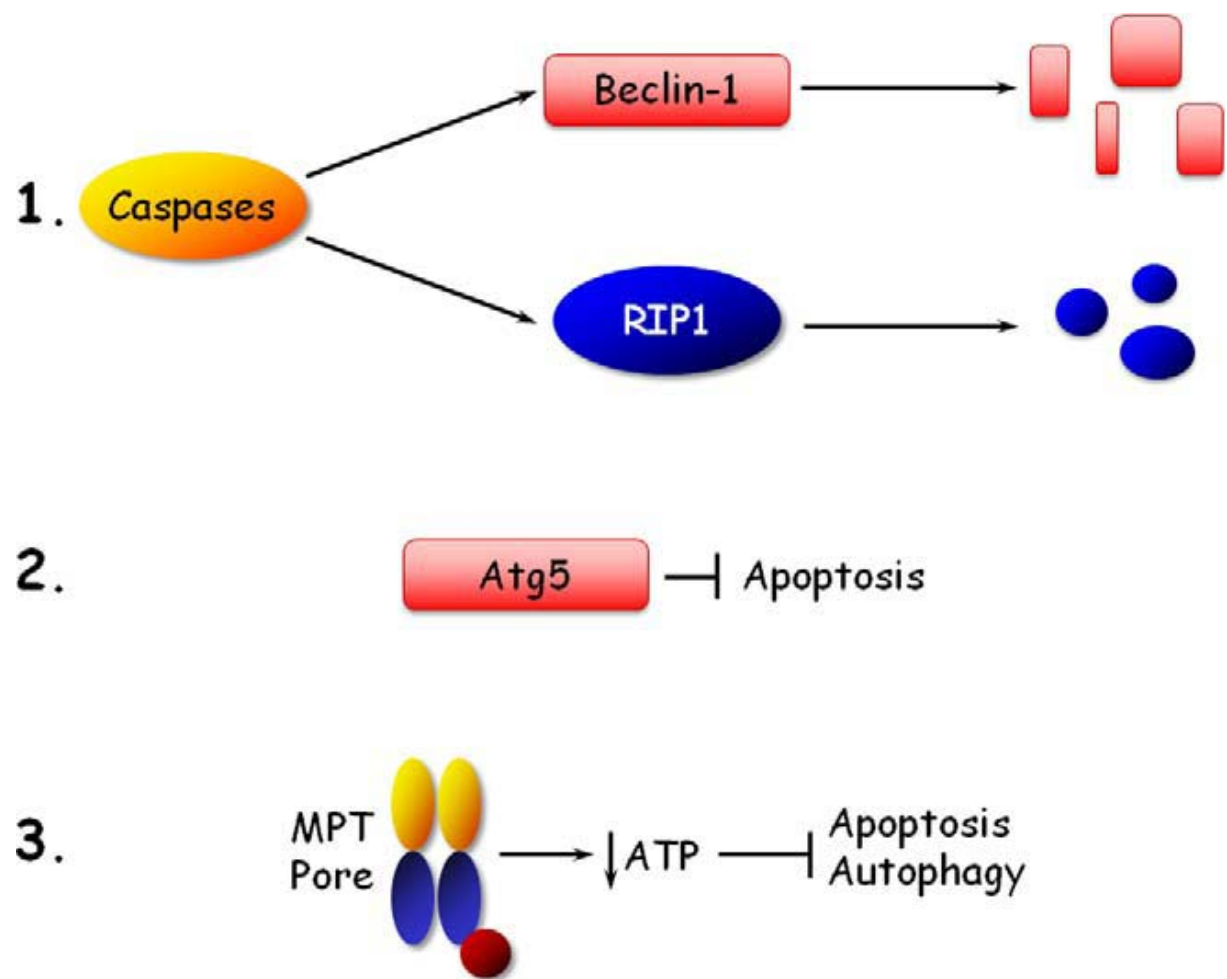


1976]. This pathway involves opening of a large conductance pore, the mitochondrial permeability transition pore (MPTP), resulting in the rapid exchange of solutes up to 1.5 kDa in size. The resulting influx of cytosolic water causes matrix expansion, leading to outer membrane rupture with the release of pro-apoptotic factors from the intermembrane space. Due to energetic collapse and uncontrolled enzymatic processes caused by matrix swelling, cell death often occurs before apoptosis can proceed [Halestrap et al., 1998]. Further studies demonstrated that the MPTP remains close during ischaemia and open only during the first few minutes of reperfusion [Griffiths et al., 1995]. Phosphorylated GSK3- $\beta$  was shown to play a pivotal role in the inhibition of MPTP [Juhaszova et al., 2004]. Some studies suggest that PI3-K/Akt may protect the myocardium against ischaemia/reperfusion injury by acting at the level of mitochondria through the activation of GSK-3 $\beta$ , thereby inhibiting the MPTP [Davidson et al., 2006]. It was also shown that PKC- $\epsilon$  protects the heart against ischaemia/reperfusion injury by inhibiting the MPTP [Costa et al., 2008].

#### **1.6.4.) Cell death cross talk in ischaemia/reperfusion**

There is a tendency to study different modalities of cell death as detached processes, but it has become more obvious that substantial cross talk occurs between autophagic, necrotic and apoptotic pathways. As indicated in **Figure 1.7**, caspases can cleave Beclin-1 and receptor-interacting protein 1 (RIP1), which are two key mediators of autophagy and apoptosis respectively. Thus, apoptosis can suppress both autophagy and necrosis [Djavaheri-Mergny et al., 2010; Lin, et al., 1999]. On the contrary, inhibition of the key autophagic protein Atg5 triggers apoptosis by a mechanism which is still unknown [Boya et al., 2005]. Opening of the pro-necrotic MPTP in the mitochondrial inner membrane, will result in adenosine triphosphate (ATP) depletion, which in turn will inhibit the induction of ATP dependent autophagy and apoptosis. Alternatively, studies indicated that inhibition of apoptosis with caspase inhibitors, will trigger autophagic or necrotic cell death [Vandenabeele et al., 2006]. Thus care must be taken in developing pharmacological agents or interventions for the treatment of ischaemia/reperfusion injury. By selectively inhibiting necrosis during reperfusion, it is possible that cells will still die, but through apoptosis and/ or autophagy instead.





**Figure 1.7:** Cross talk between apoptosis, autophagy, and necrosis. Depiction of the mechanisms by which the 3 forms of cell death can influence one another:

(1) Caspases can cleave beclin-1 and receptor-interacting protein 1 (RIP1), two key mediators of autophagy and necrosis, respectively. Thus, apoptosis can suppress the two other modes of death.

(2) The key autophagic protein, Atg5, suppresses apoptosis, although the exact mechanisms are still unknown.

(3) Opening of the pro-necrotic mitochondrial permeability transition (MPT) pore will lead to adenosine triphosphate (ATP) depletion, which in turn will inhibit the progression of both autophagy and apoptosis [Baines, 2011].

## 1.7) The importance of perfusion models in IPC

Research efforts to elucidate the mechanism of IPC have often been hampered by the opposing results obtained by different laboratories. This has been attributed to differences in species (rabbits versus rats), perfusion models (in vitro versus in vivo), the size of the ischaemic zone (global versus regional ischaemia), the duration of ischaemia or the endpoint of protection (functional recovery of infarct size). Of particular concern are the conflicting data obtained with minor differences in only the experimental model or protocol. The 2 most frequently used experimental models of the perfused rat heart are the Langendorff retrogradely perfused model and the working heart model.

The Langendorff model is based on forcing blood or a fluid into the coronary arteries through a canula implanted in the aorta [Langendorff, 1895]. It does not permit the left ventricle to eject the perfusate (cardiac output) and is therefore a non-working heart model [Doring 1988]. The Langendorff model employ regional ischaemia and use infarct size or the incidence of reperfusion-induced arrhythmias as endpoint. Protection by IPC against arrhythmias has conclusively been reported in studies using rats, but its protective effects in large animals is controversial [Ovize et al., 1995; Grund et al., 1997]. It also appears that in the same species, the triggers required to precondition the heart against necrosis and arrhythmias, may differ [Li et al., 1992]. When using infarct size as endpoint, the age of the animal is an important aspect that needs attention. Tani et al., 1997 reported that with middle age rats (50 weeks), the protective effect of IPC was reversed compared to young (12 week old ) rats. Furthermore the choice of anaesthetic agent is an important aspect, since anaesthetic agents may affect the magnitude of protection. A study by Haessler et al., 1994 showed that using identical experimental protocols, protection in pentobarbital anaesthetized animals was significantly greater than in isoflurane or ketamine/xylazine – anaesthetized animals. This observation was confirmed by later studies [Cope et al., 1997]. The duration of infarct size development is different in various species [Nienaber 1983]. The early response to ischaemia depends to a large extent on the existence of a collateral circulation [Harken et al., 1981]. Various studies have shown that differences exist in the extent of collateralization of the various species [Schaper, 1971; Schaper et al., 1967; Schaper et al., 1992]. Dogs have a

well-developed coronary collateral circulation, while collateral blood flow in pigs and baboons is extremely low [Schaper 1971; White & Bloor 1981; Shen et al., 1996; Crozatier et al., 1978]. The collateral circulation in the rat is low and the rabbit show intraspecies differences [Schaper et al., 1992; Winkler et al., 1984]. IPC has been shown in animals with [Murry et al., 1986] and without [Schott et al., 1990] a collateral circulation, implying that IPC is independent of the extent of a collateral circulation. However, collateral blood flow is a major determinant of infarct size and in order to obtain an accurate assessment of infarct size, collateral blood flow should always be determined in species with a collateral circulation. Body temperature is another important determinant of infarct size development with the magnitude of the effect depending on the duration of the coronary artery occlusion [Chien et al., 1994; Duncker et al., 1996; Schwartz et al., 1997]. The majority of studies use the ratio of infarcted area (IA) and the area at risk (AR) to assess the reduction in infarct size by IPC [Verdouw et al., 1998]. In the further analysis it then assumed that this ratio is independent of the area at risk, i.e. the relation between AR and IA is proportional. This has been shown to be true for rats [Ytrehus et al., 1993] but not for pigs [Koning et al., 1995] and rabbits [Ytrehus et al., 1993]. The linear regression line relating to infarcted area and area at risk has a zero-intercept on the AR-axis. This implies that the mathematical description of this relationship is  $IA = a \cdot AR + b$  or  $IA/AR = a + b/AR$ . Especially for small AR,  $IA/AR$  depends on AR. Thus unless AR exceeds 20% of the left ventricular mass,  $IA/AR$  should be used with care, in particular when studies are compared in which the areas at risk differ substantially [Verdouw et al., 1998].

Neely et al., 1967 was the first to develop a working performing isolated heart model some 40 years ago. The working heart model employ global ischaemia and permits the left ventricle to eject perfusate (cardiac output). It therefore uses functional recovery as endpoint. The advantage of the working heart model is that it allows construction of cardiac function curves under a wide variety of conditions, such as before and after the post-ischaemic recovery phase of the reperfusion period [Verdouw et al., 1998]. The working heart model is particularly useful for the study of metabolic pathways because the content of the substrate in the perfusion solution can be altered. The metabolic products can be determined in the effluent. Furthermore, at the end of the experiment, myocardial tissue can be

utilized for biochemical and histological analysis. When using global ischaemia one eliminates the interaction between the myocytes of different regions of the heart, which may have an impact on the ultimate function of regionally ischaemic myocardium [Fan et al., 1997].

Many laboratories in the field of IPC use infarct size as endpoint. Reduction in infarct size is regarded by many as the “gold standard” for the evaluation of the protection afforded by IPC [Yellon et al., 1998]. It is however, not always associated with an improvement in functional recovery in rabbits, dogs and pigs in vivo [Schulz et al., 2001]. Accurate measurement of infarct size can be complicated by the choice of staining and the degree of collateral flow [Shirato et al., 1998]. Functional recovery of the isolated working rat heart subjected to global ischaemia has been the method of choice in many studies [Cave & Hearse 1992; Csonka et al., 1999; Goto et al., 1992; Volovsek et al., 1992]. A study by Lochner et al., 2003 showed that IPC-induced improved functional recovery was best demonstrated in the globally ischaemic working heart. Improved functional recovery was seen at all ischaemic time intervals studied in this model. It was only seen in one series of non-working retrogradely perfused hearts after 30 min global ischaemia.

The majority of studies focus on only one relatively early time point of reperfusion and this has led to the dismissal of cell death during late-stage reperfusion. This highlight the need for studies investigating longer reperfusion times in order to ascertain whether a reduction in infarct size is associated with a decrease in cell death during reperfusion. Therefore, we need better longer-term studies examining the different modalities of cell death that occur during late-stage reperfusion, to be able to understand their role and contribution to cardiac dysfunction and the underlying mechanisms that drive them.

In view of the above, the overall aims of the present study were:

- 1) to characterize the activation patterns of the various **kinases** at different time points during reperfusion after IPC and to compare it with non-preconditioned hearts (Non-IPC) at the same time points during reperfusion after global ischaemia;
- 2) to assess **autophagy** at these different time points during reperfusion after IPC and to compare it with non-IPC hearts at the same time points during reperfusion after global ischaemia;
- 3) to assess **apoptosis** after 30 minutes of reperfusion after IPC and to compare it with non-IPC hearts at the same time point during reperfusion after global ischaemia;
- 4) to identify the **underlying mechanisms involved** contributing to poor functional recovery of the IPC hearts during early (15 min) reperfusion, by employing 2D proteomics;
- 5) to assess whether activation of the various **kinases** and **heat shock proteins** **correlate** with functional recovery and/or other haemodynamic parameters of the heart; i) before and after sustained ischaemia, ii) after the IPC protocol (3xIPC) and also iii) after IPC during early (5 min and 10 min ) and late (30 min) reperfusion;
- 6) to identify the **kinase(s)** of which the activation patterns do correlate with functional recovery of the ischaemic heart after IPC during 30 min reperfusion.

## Chapter 2

### Materials and Methods:

#### 2.1) Animals

Male Wistar rats weighing 250 -300 gram ( $\pm$  8 weeks of age) were used in all experiments. Animals were cared for in the Central Animal Facility (CRF), which is an “Association for Assessment and Accreditation of Laboratory Animal Care” (AAALAC ) accredited unit, until the day of experimentation. Rats were housed in a thermally controlled environment ( $\pm$  21°C) and had free access to food and water. The study (project number: **P09/05/009**) has been approved by the Research Ethics Committee: Animal Care and Use (REC: ACU) of the University of Stellenbosch.

#### 2.2) Isolated working heart perfusions (Fig. 2.1)

Rats were randomly assigned to different experimental groups. Rats were anaesthetized by intraperitoneal injection with sodium pentobarbitone, at a dosage of 120 mg/kg body mass, i.e. approximately 30 mg/rat. Hearts were excised and arrested in cold (4°C) Krebs-Henseleit Buffer containing 119 mM NaCl, 25 mM NaHCO<sub>3</sub>, 4.75 mM KCl, 1.185 mM KH<sub>2</sub>PO<sub>4</sub>, 0.6 mM MgSO<sub>4</sub>, 0.6 mM NaSO<sub>4</sub>, 1.25 mM CaCl<sub>2</sub>.2H<sub>2</sub>O, 10 mM glucose. The buffer was gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> prior to and during the perfusion protocol. Isolated rat hearts were rapidly mounted on the aortic cannula of the Neely-Morgan working heart perfusion system and retrogradely perfused through the aorta at a preload of 100 cm H<sub>2</sub>O for 15 min.

After cannulation of the aorta, the left atrial cannula was inserted into the pulmonary vein to allow atrial perfusion at a preload of 15 cm H<sub>2</sub>O. Myocardial temperature was closely monitored for the duration of the whole experiment by placing a temperature probe inside the coronary sinus. Subsequent to the stabilization period of 15 min, the mode of perfusion was changed to working heart mode for 15 min. In the working heart mode, buffer enters the heart through

the left atrium through the pulmonary vein and is ejected by the left ventricle against a hydrostatic pressure of 100 cm H<sub>2</sub>O (afterload). This was followed by a retrograde perfusion episode of 30 min. Hearts were then subjected to global ischaemia as end-point of ischaemic damage.

## **2.2.1) Global ischaemia**

Hearts were subjected to 25 min global ischaemia, (cessation of perfusate flow to the whole heart). Global ischaemic temperature was maintained at 36.5°C. This was followed by 10 min retrograde reperfusion and 20 min working heart mode reperfusion.

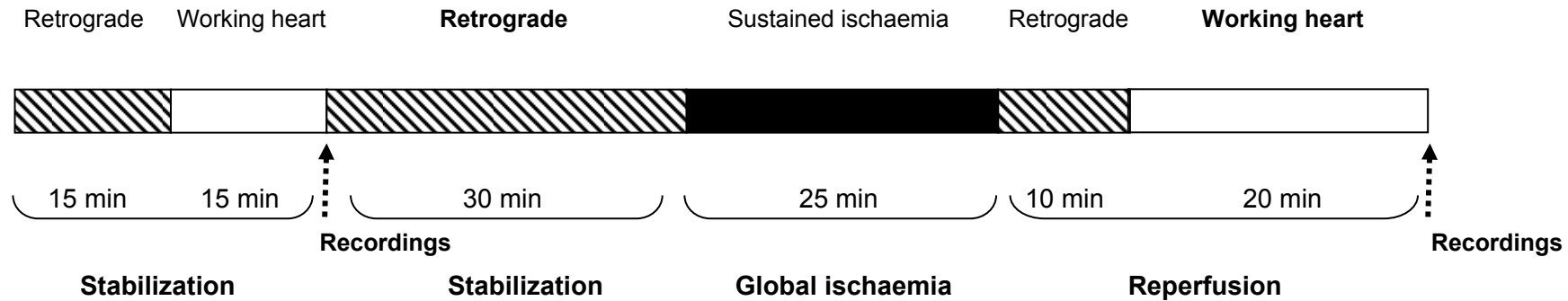
### **2.2.1.1) Mechanical functional recovery after global ischaemia**

Mechanical functional recovery was measured as an end-point in hearts subjected to global ischaemia. Coronary (Q<sub>e</sub>) and aortic (Q<sub>a</sub>) flow rates in ml/min were measured manually. A side branch of the aortic cannula was connected to a Viggo-spectramed pressure transducer, in order to obtain the aortic pressure (mmHg). The heart rate (HR), peak systolic pressure (PSP), diastolic pressure, mean blood pressure and ejection fraction (EF), were obtained from the recordings made. The maximum rate of left ventricular pressure (LVP) rise, (dP/dT<sub>max</sub>) and fall (-dP/dT<sub>min</sub>) were also obtained from the recordings made. The following parameters were calculated:

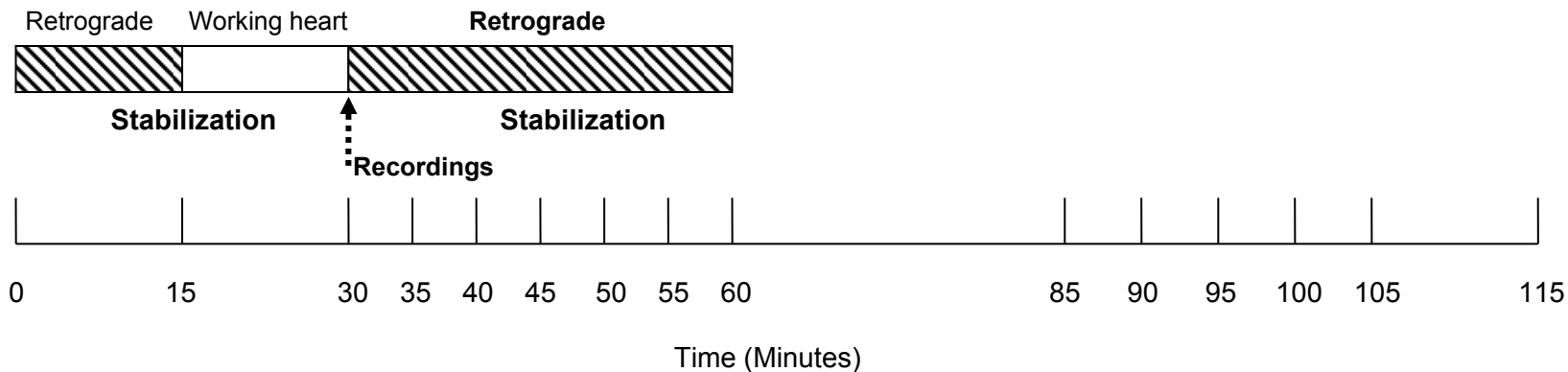
- Cardiac output (CO) in ml/min = Q<sub>a</sub> + Q<sub>e</sub>
- Stroke volume (SV) ml/min = CO / HR
- The mean external power (TW) produced by the left ventricle in mWatts = 0.002222(PAO – 11.25) (CO) where PAO = aortic pressure and CO = cardiac output (Kannengieser, 1979).

These measurements were made before global ischaemia (during the stabilization phase) and after global ischaemia, during reperfusion, as indicated by the arrows in Fig. 2.1. Functional recovery of hearts was determined after 30 min reperfusion, by expressing post-ischaemic aortic output as a percentage of pre-ischaemic aortic output.

### Basic perfusion protocol:



### Controls:



**Figure 2.1** Basic perfusion protocol for the isolated perfused working rat heart with global ischaemia. Mechanical functional recordings, were made after 15 min working heart and after 30 min reperfusion, as indicated by the dashed arrows (↑). For control hearts, functional recordings were made after 15 min working heart as indicated by the dashed arrows (↑).



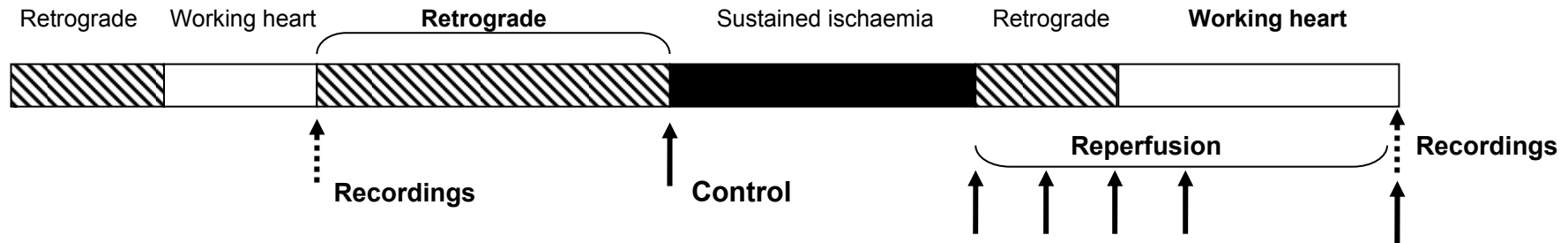
### 2.2.1.2) Experimental Protocol (Fig. 2.2)

**(i) Controls:** Hearts were stabilized for 30 min (15 min retrograde, 15 min working heart). Functional recordings were made after 15 min working heart (as indicated by the dashed arrows in Fig. 2.1 and Fig. 2.2) and hearts were retrogradely perfused for another 30 min. Hearts were then snap-frozen before sustained ischaemia for Western blotting analysis, as indicated by the solid arrows (Fig.2.2). Control hearts were thus not subjected to ischaemia/reperfusion or the IPC protocol, but perfused under oxygenated conditions.

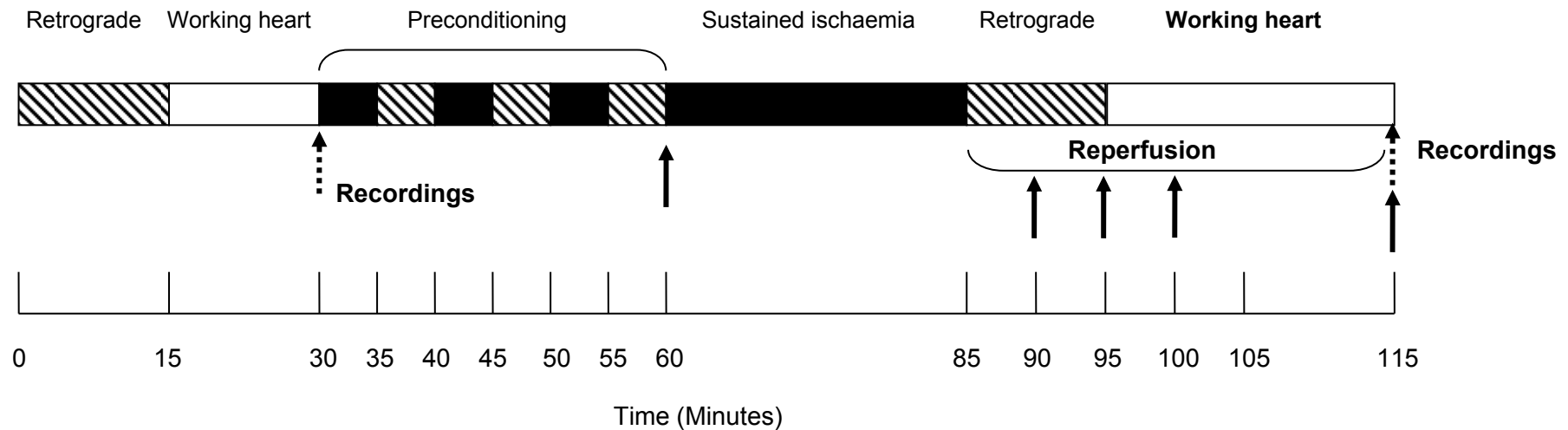
**(ii) Non-preconditioning (Non-IPC):** Hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde). Functional recordings were made at 15 min working heart as indicated by the dashed arrows in Fig. 2.2. Hearts were subsequently subjected to 25 min sustained global ischaemia followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, functional recordings were made (dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows (Fig. 2.2). Alternatively, when investigating shorter reperfusion times, hearts were snap-frozen at 5, 10 and 15 min reperfusion respectively, as indicated by the solid arrows in Fig. 2.2.

**(iii) Ischaemic preconditioning (IPC):** Hearts were stabilized for 30 min (15 min retrograde, 15 min working heart). Functional recordings were made after 15 min working heart as indicated by the dashed arrows in Fig. 2.2. Hearts were then preconditioned by 3x5 min global ischaemia, alternated with 5 min reperfusion. Hearts were subjected to 25 min sustained global ischaemia, followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, functional recordings were made (dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows (Fig. 2.2). Alternatively when investigating shorter reperfusion times, hearts were snap-frozen at 5, 10 and 15 min reperfusion respectively, as indicated by the solid arrows in Fig. 2.2.

### Non-preconditioned hearts:



### Ischaemic preconditioned hearts:



**Figure 2.2** Experimental protocol according to a timeframe indicating at which time intervals, indicated by the solid arrows (↑), hearts were snap-frozen for western blot analysis. Functional recordings were indicated by the dashed arrows (⋮↑).

## 2.3) Western Blot analysis

Hearts were snap-frozen at different time intervals as indicated in Fig.2.2 and stored in liquid nitrogen, before the preparation of tissue lysates for Western blotting analysis. The following phosphorylated- and appropriated total primary antibodies (Cell Signaling Technology, Massachusetts, United States) were used: phospho-p38 MAP Kinase (Thr 180/Tyr 182) and total p38 MAP Kinase; phospho ERK (p44/42) MAP Kinase (Thr 202/Tyr 204) and total ERK (p44/42) MAP Kinase; phospho PKB/Akt (Ser 473) and total PKB/Akt; phospho HSP-27(Ser 82) and total HSP-27 (Rodent Preferred); HSP-70 (D69); phospho CREB (Ser 133) and total CREB (48H2); phospho-CaMKII (Thr 286) and total CaMKII (pan); phospho AMPK $\alpha$  (Thr 172) and total AMPK $\alpha$ ; phospho GSK-3 $\beta$  (Ser9) and total GSK-3 $\beta$ ; Caspase-3; Cleaved PARP (Asp 214) rat specific antibody, Beclin-1 and LC3B. Equal protein loading was assessed by probing membranes with  $\beta$ -Tubulin antibody (Cell Signaling Technology, Massachusetts, United States). ECL anti-rabbit IgG horseradish peroxidase-linked species-specific whole antibody (from donkey) was used as secondary antibody (Amersham, GE Healthcare, UK.) Protein bands obtained were analyzed using densitometry (UNSCAN-IT Software version 5.1; Silk Scientific Corporation, United States).

### 2.3.1) Preparation of lysates from heart tissue

Approximately 30  $\mu$ g of tissue in 900  $\mu$ l of lysis buffer containing 20 mM Tris-HCl, pH 7.5; 1 mM EGTA; 1 mM EDTA; 150 mM NaCl, 1 mM  $\beta$ -glycerophosphate; 2.5 mM sodium pyrophosphate; 1mM sodium vanadate; 1% (v/v) Triton-X100; 10  $\mu$ g/ml leupeptin; 10  $\mu$ g/ml aprotinin and 50  $\mu$ g/ml phenylmethylsulfonylfluoride (PMSF) was used for the preparation of tissue lysates. Tissues were homogenized with a politron for 2 x 5 sec, maintaining the temperature at 4°C throughout all procedures, after which it was transferred to microcentrifuge tubes. It was centrifuged at 14 500 rpm for 10 min at 4°C and the resulting supernatant (total cell lysate) was transferred to a new tube. The pellet was discarded. The Bradford assay (Bradford, 1976) was used for determining the protein content of the samples. The protein concentration of all samples were adjusted and equalized to 25  $\mu$ g/ 9  $\mu$ l with lysis and sample buffer. Samples were boiled for 5 min and stored overnight at -80°C.

### **2.3.2) Electrophoresis**

Polyacrylamide gels, consisting of a 4% stacking gel and 12% separation gel, were prepared by the method of Laemmli (Laemmli, 1970). Pageruler (Thermo Scientific, Bremen, Germany) was used as molecular weight marker. Samples were subjected to electrophoresis, using the standard Bio-Rad Mini Protean III system (Bio-Rad Laboratories Inc. Hercules, CA, United States). Gels were run at 100 V for 10 min and a further 50 min at 200V. Alternatively, 4-15% Criterion™ TGX™ precast gels (midi-gels) were used, using the Bio-Rad Criterion gel system (Bio-Rad Laboratories, Inc. Hercules, CA, United States). Electrophoresis was carried out at 200 V for 50 min, using the Laemmli system with electrophoresis buffer containing 25 mM Tris, 200 mM Glycine and 0.1% (w/v) SDS (Laemmli, 1970).

### **2.3.3) Western Blotting**

The separated proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane (Merck Millipore, Massachusetts, United states). The blotting step was carried out at 200A for 60 min with cooling, using the Bio-Rad Mini Trans-blot Cell (Bio-Rad Laboratories, Inc. Hercules, CA, United States). Alternatively, the blotting step for the Criterion (midi-gels) were carried out at 100V for 35 min with cooling, using the Criterion blotter with plate electrodes. Proper transfer and equal protein loading was assessed by staining the membrane with 5% (w/v) Ponceau-S. Non specific protein binding sites on the membrane were blocked with 5% (w/v) fat free milk in TBSTween (Tris buffered saline with 0.1% (v/v) Tween 20) for 2 hours at room temperature. Membranes were incubated with the primary antibody (phosphorylated or total antibody) of interest, overnight at 4°C with gentle agitation. After thorough washing with TBSTween, membranes were incubated with the secondary antibody (Rabbit IgG Horseradish Peroxidase-linked; Amersham, GEHealthcare, UK), for 60 min at room temperature.

### **2.3.4) Chemiluminescence**

After thorough washing of membranes with TBSTween, membranes were incubated with the chemiluminescent reagents (ECL detection system with luminol; Amersham, GE Healthcare, UK.) for 1 min at room temperature in the dark. Membranes were exposed to ECL Hyperfilm and a hypercassette (Amersham, GE Healthcare, UK), to visualize protein bands. Densitometry was used to scan and quantify the protein bands in order to perform the statistical analysis.

### **2.4) Statistical analysis**

The total number of animals included in the study were 234. For perfusions, inclusion criteria applied were: AO of 36 ml/min and above; CF of 20 ml/min and below. Exclusion criteria applied were: AO below 36 ml/min; CF above 20 ml/min.

For multiple comparisons one-way analysis of variance (ANOVA) was used with the Bonferroni correction as post-hoc test (GraphPad Prism software version 5.0). For perfusions and western blotting analysis a minimum of 8 animals were used per experimental group and a maximum of 4 groups were included in each comparison. When 2 groups were included in a comparison, data were analyzed using the independent Student t-test. Results obtained were expressed as mean  $\pm$  standard error of the mean (SEM). For correlation studies the Pearson test with a two tailed p value was utilized. Results were expressed as the Pearson correlation coefficient (r). A p value of  $<0.05$  was considered significant in all the statistical analysis performed.

## **2.5.) Two- dimensional (2D) Proteomics**

### **2.5.1) Isolation of the mitochondrial fraction from heart tissue**

Mitochondria were isolated from Non-IPC and IPC hearts at the end of 15 min reperfusion, as indicated in figure 2.2, using a mitochondrial isolation kit (Sigma-Aldrich St. Louis, Missouri, United States). This kit enables the fast isolation of an enriched mitochondrial fraction for proteomic studies [Lopez, 2000; Rabilloud, 1998]. The isolation procedure was performed at 4°C.

Approximately 120 mg tissue was weighed in an Eppendorf tube and cut with the aid of a scalpel and glass plate to even smaller slices. Tissues were suspended in 10 volumes of extraction buffer A (10 mM HEPES, pH 7.5, 200 mM mannitol, 70 mM sucrose and 1 mM EGTA) containing 0.25 mg/ml trypsin in a 2 ml Eppendorf tube and incubated on ice for 3 minutes, followed by centrifugation (cooled Eppendorf centrifuge) for 15 seconds at 13 400 rpm. The supernatant was removed by aspiration and 8 volumes of extraction buffer A containing 0.25 mg/ml trypsin was added to the tissue and incubated on ice for 20 minutes. An albumin solution was then added to a final concentration of 10 mg/ml to quench the proteolytic reaction followed by centrifugation for 15 seconds at 13 400 rpm. The supernatant was removed by aspiration and the pellet washed with 8 volumes of extraction buffer A. The tissue was centrifuged for 15 seconds at 13 400 rpm and the supernatant removed by aspiration. Eight volumes of extraction buffer A was added to the tissue, followed by homogenization using a 3 ml volume homogenizer (Potter-Elvehjem). Total homogenization of the tissue was ensured by moving the pestle up and down 20-30 times. The sample was centrifuged at 1000xg for 5 minutes, the supernatant removed and transferred to a new tube followed by centrifugation at 3500xg for 10 minutes. The mitochondrial pellet was resuspended in storage buffer containing 10 mM HEPES, pH 7.4, containing 250 mM sucrose, 1mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K<sub>2</sub>HPO<sub>4</sub> and 1 mM DTT. The protein concentration of the mitochondrial preparation was determined by the Quick Start™ Bradford Protein assay (Sedmak and Grossberg, 1977) with bovine serum albumin as standard (Bio-Rad Laboratories, Inc. Hercules, CA, United States).

### 2.5.2) Isolation of the cytosolic fraction of heart tissue

The cytosolic fraction of Non-IPC and IPC hearts were isolated at the end of 15 min reperfusion, as indicated in figure 2.2, using the Calbiochem ProteoExtract Subcellular Proteome Extraction kit (Merck, Darmstadt, Germany). This kit is designed for the reproducible extraction of subcellular proteomes from mammalian cells to yield 4 sub-proteome fractions which are enriched in cytosolic, membrane/organelle, nuclear and cytoskeletal proteins. The **cytosolic** fraction was used for the 2D proteomic study.

### 2.5.3) Sample preparation prior to 2D Electrophoresis

The ReadyPrep 2D CleanUp kit (Bio-Rad Laboratories Inc. Hercules, CA, United States), was used to concentrate the **mitochondrial** and the **cytosolic** fractions respectively and also to remove (by washing away) compounds which interfered with the isoelectric focusing (IEF) step. After precipitation, the proteins were washed and then resuspended in the IEF/2D-compatible sample buffer containing 8M urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (w/v) Bio-Lyte 3/10 ampholyte, 0.002% (w/v) bromophenol blue. Protein concentration was determined using the RC/DC Assay (Bio-Rad laboratories Inc, Hercules, CA, United states). This assay is based on the Lowry assay but has been modified to be reducing agent compatible (RC) as well as detergent compatible (DC).

### 2.5.4) Two-dimensional (2D) Gel Electrophoresis

Separation in the first dimension, using Iso-electric focusing (IEF), was performed using the PROTEAN- IEF cell (Bio-Rad Laboratories Inc. Hercules, CA, United States). Samples containing 100 µg mitochondrial and 150 µg of cytosolic proteins respectively, were loaded on 11cm immobilized pH gradient strips, pH 5-8 (Bio-Rad laboratories, Inc. CA, United States).

Three samples (n=3 animals) from each group (IPC) were run in triplicate against its respective control (Non-IPC). Two comparisons were done namely, (1) the mitochondrial fraction of IPC hearts were compared to the mitochondrial fraction of Non-IPC hearts (control). This experiment was repeated twice (n=3

experiments). Therefore a total number of 6 animals (18 strips/gels) in 3 different experiments were used for the mitochondrial proteomic study. (2) the cytosolic fraction of IPC hearts were compared to the cytosolic fraction of Non-IPC hearts (control). This experiment was repeated twice (n=3 experiments). Therefore a total number of 6 animals (18 strips/gels) were used in 3 different experiments for the cytosolic proteomic study.

Strips were rehydrated under passive conditions for 12 h at 20 °C and focused at 200 V for 20 min. The voltage was linearly increased over 2h to a maximum of 8000 V and then run (rapid ramping) to accumulate a total of 40 000 V/h.

Prior to the second dimension, the immobilized pH gradient strips were first equilibrated for 15 min in 0.375 M Tris-HCL, pH 8.8, 6M urea, 2% (w/v) SDS, 20% (v/v) glycerol and 2% (w/v) DTT, followed by equilibration for another 15 min in 0.375 M Tris-HCL, pH 8.8, 6M urea, 2% (w/v) SDS, 20% (v/v) glycerol and 2.5% (w/v) iodoacetamide.

Strips were embedded in 0.5% (w/v) low melting point agarose containing 0.003% (w/v) bromophenol blue on the top of Criterion XT 4-12% precast Bis-Tris gels (Bio-Rad Laboratories, Inc. Hercules, CA, United states), containing a 4% stacking gel. The PeqGold protein marker II (Optima Scientific, Mississauga, Canada), was used as molecular weight ladder. Electrophoresis was performed at 200V, constant for 55 min. Gels were fixed in 40% (v/v) methanol and 7% (v/v) acetic acid for 60 min at room temperature and stained overnight with Brilliant Blue G-Colloidal Concentrate (Sigma-Aldrich, St Louis, Missouri, United States) to visualize protein spots. Gels were subjected to a rapid destaining step, using 40% (v/v) methanol and 10% (v/v) acetic acid for 60 sec at room temperature, followed by further destaining in 25% (v/v) methanol.

### **2.5.5) Identification of differentially expressed mitochondrial and cytosolic proteins spots**

Gels were scanned with a GS-800 Calibrated Densitometer, using the Quantity One-4:5.2 (basic) software program (Bio-Rad Laboratories Inc. Hercules, CA, United States) to obtain gel images. The gels were washed with milli-Q water and



stored in 25% (w/v) ammonium sulphate until spots were excised. The gel images were analyzed using the PDQuest advanced version 8.1.1 software program (Bio-Rad Laboratories, Inc. Hercules, CA, United States). As this software program has a built-in statistical program, it was able to identify protein spots, that differ significantly in intensity between the three sets of triplicate gels (3x IPC mitochondrial versus 3x Non-IPC mitochondrial) and (3x IPC cytosolic versus 3x Non-IPC cytosolic), respectively. It only took spots in consideration that were present on all gels. This was done to exclude any possible technical error or artefacts on the gels. Only spots that differed significantly ( $p < 0.05$ ) between the IPC and Non-IPC gels, as identified by PD Quest, were manually excised and prepared for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis. These spots (spots of interest), were manually excised using a razor blade, cutting a section of gel no greater than 0.5 cm x 0.15 cm, which was then sliced into five equally sized pieces and placed in a 96-well plate.

### **2.5.6) Trypsin digestion of mitochondrial and cytosolic proteins**

All reagents were prepared immediately prior to use. The acetonitrile was HPLC grade and the other reagents were the highest possible commercial grade available. Milli-Q water or 17 megaohm/cm Nanopure water was used in all components of the procedure. The MassPREP station (Waters MS Technologies, Manchester, UK ) in the Proteomics Facility , University of Warwick, was used for this method.

Gel plugs were washed twice with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate and when fully destained rinsed with 100% (v/v) acetonitrile. Gel plugs were air dried for 10 min and proteins reduced for 30 min with 10 mM dithiothreitol (DTT), followed by alkylation with 55 mM iodoacetamide. Gel plugs were rinsed with 100% (v/v) acetonitrile and then 100 mM ammonium bicarbonate followed by 100% (v/v) acetonitrile for a further three times.

For the in-gel digestion, a 25  $\mu$ l aliquot of 6 ng/ $\mu$ l trypsin (Promega Corporation, Wisconsin, Madison, United States) was added to each sample and allowed to incubate at 37°C for 4.5 hours. An aqueous solution of 30  $\mu$ l, containing 2% (v/v) acetonitrile and 1% (v/v) formic acid was used to extract the resulting peptides.

The extracted peptides were placed in a cooled 96-well plate, while the second extraction was performed. For the second extraction, 15 µl of an aqueous solution containing 51% (v/v) acetonitrile and 0.5% (v/v) formic acid was used. The second extract was combined with the first extract in a cooled second 96-well plate and stored at -80°C prior to analysis by mass spectrometry.

### **2.5.7) Peptide separation by in-line liquid chromatography (LC) and electrospray ionisation (ESI) mass spectrometry**

The 96-well plate containing the extracted tryptic peptides was transferred to a Micromass modular CapLC and autosampler system (Waters MS Technologies, Manchester, UK ). LC solvents were supplied by Mallinckrodt Baker, Inc. A 6.4 µl aliquot of each extract was mixed with 13.6 µl of 0.1% formic acid and loaded onto a 0.5 cm LC Packings C18, 5 µm, 100 A 300 µm id µ-precursor cartridge. (Dionex Corporation, Sunnyvale, CA, United States). The following LC conditions were used: Solution A [95% water; 4.9% (v/v) acetonitrile and 0.1% (v/v) formic acid] and Solution B [4.9% water; 95% (v/v) acetonitrile and 0.1% (v/v) formic acid]. Flushing the column with solution A desalted the bound peptides before a linear gradient of solution B at a flow rate of 200 nl min<sup>-1</sup> eluted the peptides for further resolution on a 15 cm LC Packings C18, 5 µm 5 A 75 µm i.d. PepMap analytical column (Dionex Corporation, Sunnyvale, CA, United States). The gradient separations were 0.10 min (95% A: 5% B); 3 min (95% A: 5% B); 27.5 min (60% A: 40% B); 28 min (5% A: 95% B); 31 min (5% A: 95% B); 31.5 min (95% A: 5% B); 40 min (95% A: 5% B). The eluted peptides were analysed on a Micromass Quadrupole Time of Flight (Q-TOF) Global Ultima mass spectrometer (Waters MS Technologies, Manchester, UK ) fitted with a nano-LC sprayer with an applied capillary voltage of 3.5 kV. The instrument was calibrated against a collisionally induced decomposition (CID) spectrum of the doubly charged precursor ion of [glu<sup>1</sup>]-fibrinopeptide B, (GFP); Sigma-Aldrich, St Louis, Missouri, United States. A calibration was accepted when the error obtained on all subsequent acquisitions was < 20 ppm.

Sensitivity was assessed by the detection of a 500 fmol injection of GFP, with a base peak signal:noise ratio of > 50:1 on the doubly charged ion. The sensitivity and calibration of the MS instrument, as well as the chromatographic resolution of

the GFP peak, were checked at regular intervals during the analysis. The instrument was operated in data dependent acquisition (DDA) mode over the mass/charge ( $m/z$ ) range of 50-2000. During the DDA analysis, both MS and tandem mass spectrometry (CID) was performed on the most intense peptides as they eluted from the column.

#### **2.5.8) Data analysis:**

The uninterpreted data from the MS analysis were processed using the Micromass ProteinLynx Global Server version 2.3 software package (smoothed, background subtracted, centred and deisotoped). The processed data was mass corrected against the doubly charged GFP peptide and Peak list files (PKL's) were created. The resulting Micromass PKL files were subjected to the MASCOT search engine ([www.matrixscience.com](http://www.matrixscience.com)), using MS/MS Ion search and the SwissProt database to identify proteins. Mammalia was used as subspecies and other search parameters specified for were: carbamidomethylated at cysteine residues (fixed modifications), oxidized at methionine residues (variable modifications) and 1 missed trypsin cleavage was allowed for. Peptide tolerance was set at 0.2 Da and MS/MS tolerance was 0.5 Da; peptide charge was set at +2 and +3. All protein identifications were significant according to the probability-based MOWSE scores, which were reported as  $-10 \times \log_{10}(p)$ , where  $p$  is the probability that the observed score is a random event.

#### **2.5.9) Statistical analysis:**

A total number of 12 animals were used in the 2D proteomic study. 2D gels were analyzed by the PDQuest software program, version 8.1.1. (Bio-Rad Laboratories, Inc Hercules, CA, United States). The replicate group analysis was performed with Non-PC (triplicate gels) as control group and IPC (triplicate gels) as experimental group. The significance level was set as  $p < 0.05$  and data was presented in terms of fold decrease or fold increase compared to control. The Mascot search engine was used to identify peptide protein matches. The search algorithm uses the probability-based MOWSE scoring algorithm (Pappin et al., 1993). Mascot reports a probability-based Ion Score for each peptide match, which indicates the statistical significance of the MS/MS spectral assignment.

Individual ion scores >39 indicate identity or extensive homology ( $p < 0.05$ ) and a positive identification was defined and reported when the MOWSE scores (>40) were significant ( $p < 0.05$ ).

## Chapter 3

### Investigating the involvement of kinase activation and expression in IPC during reperfusion

#### 3.1) Introduction

The isolated perfused rat heart is a very important tool in experimental heart research. The signal transduction pathways activated by the preconditioning stimulus during the pre-ischaemic phase have been extensively delineated [Yellon & Downey, 2003]. Currently, it is proposed that the secretion of autocoids such as adenosine and bradykinin in response to the preconditioning ischaemic episodes, bind to their respective receptors on the cardiomyocytes and trigger a signaling cascade which terminates at the level of the mitochondria [Costa et al., 2005].

Emerging studies suggest that events occurring during the post-ischaemic myocardial reperfusion phase may however, underlie the cardioprotection elicited by IPC [Hausenloy et al., 2005]. In this regard, IPC has been demonstrated to modify events occurring during the reperfusion phase such as activating the pro-survival kinases of the RISK pathway [Hausenloy et al., 2005; Solenkova et al., 2006], which include PKB/Akt, a downstream effector of the PI3-kinase pathway and ERK 1/2, a member of the mitogen activated protein kinase family (MAPK).

Research efforts to elucidate the mechanisms whereby IPC protects the heart have been hampered by the opposing results obtained by different research groups. This phenomenon has been attributed to differences in species (rabbits vs rats vs mice), models (Langendorff vs working heart mode), experimental protocols (single vs multi-cycle) and severity of ischaemia (global vs regional). Of major concern are the conflicting data regarding kinase activation in the same species and model. Furthermore, researchers often report only one time point of kinase activation during reperfusion and then associate it with protection. We, therefore, hypothesized that kinase phosphorylation at reperfusion after IPC is a

time dependent, dynamic process which correlates with different functional parameters.

The aims of this part of the study therefore were:

1. to characterize the activation patterns of the various **kinases** at different time points during reperfusion after IPC and to compare it with non-IPC hearts at the same time points during reperfusion after global ischaemia
2. to assess **autophagy** at these different time points during reperfusion after IPC and to compare it with non-IPC hearts at the same time points during reperfusion after global ischaemia
3. to assess **apoptosis** after 30 minutes of reperfusion after IPC and to compare it with non-IPC hearts at the same time point during reperfusion after global ischaemia

### 3.2) Methods

To establish the involvement of the different protein kinases in IPC during early (5, 10, 15 min) and late (30 min) reperfusion, the following experimental protocol was used:

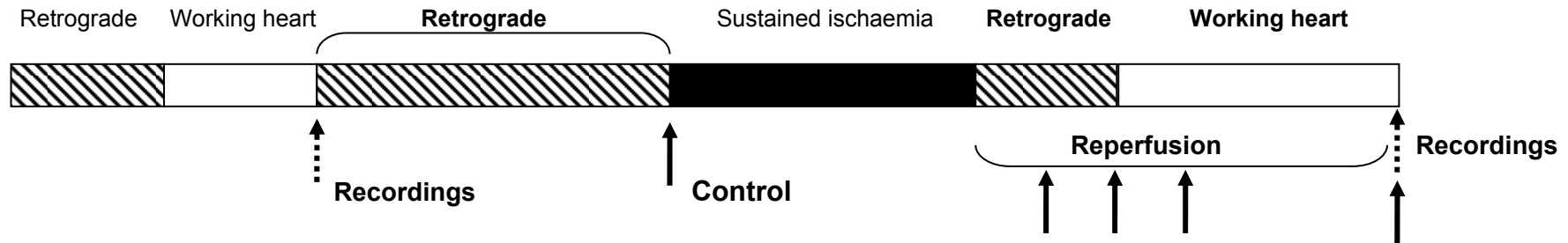
**(i) Controls:** Hearts were stabilized for 30 min (15 min retrograde, 15 min working heart). Functional recordings were made after 15 min working heart (as indicated by the dashed arrows in Fig. 2.1 and Fig. 2.2, Materials and Methods section, Chapter 2) and Fig. 3.1. Hearts were retrogradely perfused for another 30 min. Hearts were then snap-frozen before sustained ischaemia for Western blotting analysis, as indicated by the solid arrows indicated in Fig.2.2 and Fig.3.1. Control hearts were thus not subjected to ischaemia/reperfusion or the IPC protocol, but perfused under oxygenated conditions. Western blotting data of all proteins were normalized and expressed as fold change to control. The results obtained for the different phosphoproteins were normalized against the results obtained for the corresponding total protein, with the exception of the 15 min reperfusion timepoint. Equal loading was controlled for by performing ponceau staining on each membrane or blotting for  $\beta$ -Tubulin on each set of lysates per timepoint.

**(ii) Non-preconditioning (Non-IPC):** Hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde). Functional recordings were made at 15 min working heart as indicated by the dashed arrows in Fig. 2.2 and Fig. 3.1. Hearts were subsequently subjected to 25 min sustained global ischaemia followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, functional recordings were made (dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows in Fig. 2.2 and Fig. 3.1. Alternatively, when investigating shorter reperfusion times, hearts were snap-frozen at 5, 10 and 15 min reperfusion respectively, as indicated by the solid arrows in Fig. 2.2 and Fig.3.1.

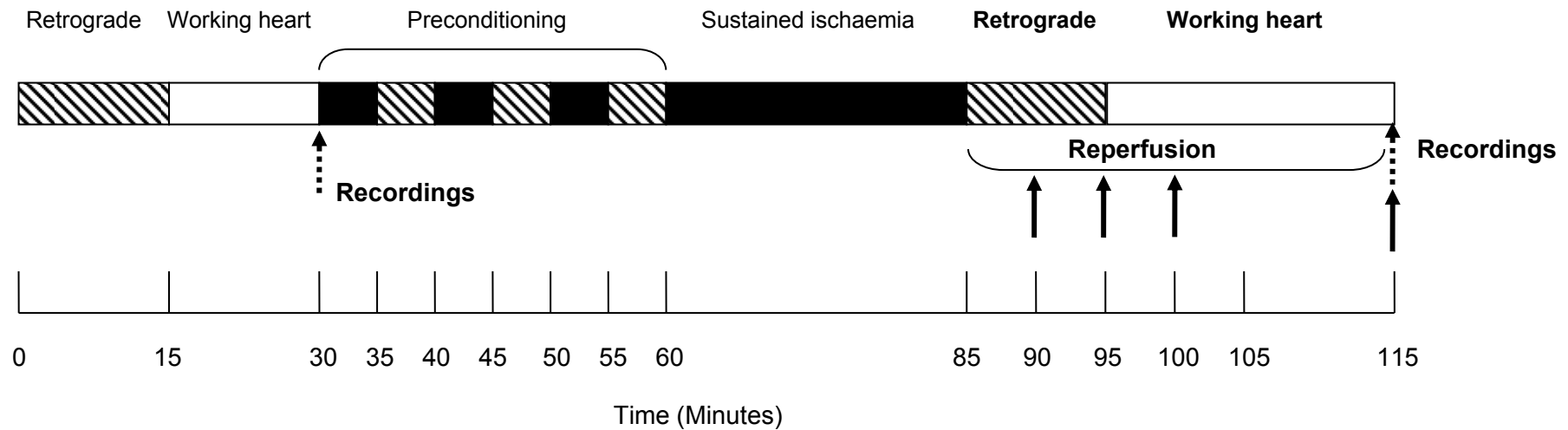
**(iii) Ischaemic preconditioning (IPC):** Hearts were stabilized for 30 min (15 min retrograde, 15 min working heart). Functional recordings were made after 15 min working heart as indicated by the dashed arrows in Fig. 2.2 and Fig.3.1. Hearts were then preconditioned by 3x5 min global ischaemia, alternated with 5 min reperfusion. Hearts were subjected to 25 min sustained global ischaemia, followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, functional recordings were made (dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows in Fig. 2.2 and Fig.3.1. Alternatively when investigating shorter reperfusion times, hearts were snap-frozen at 5, 10 and 15 min reperfusion respectively, as indicated by the solid arrows in Fig. 2.2 and Fig.3.1.

### Experimental protocol: (Fig.3.1)

#### Non-preconditioned hearts:



#### Ischaemic preconditioned hearts:



**Figure 3.1** Experimental protocol according to a timeframe indicating at which time intervals, indicated by the solid arrows (↑), hearts were snap-frozen for western blot analysis. Functional recordings were indicated by the dashed arrows (⋮).



### 3.3) Results

**Table 3.1** The haemodynamic parameters of isolated rat hearts after 15 min working heart (before sustained ischaemia)

Preconditioned hearts ( protected)													
Sample	AO (ml/min)	CF (ml/min)	CO (ml/min)	Heart rate (beats/min)	Diastole (mmHg)	PSP (mmHg)	Mean BP (mm Hg)	Total work (mW)	Ejection fraction	dP/dTmax	dP/dTmin	Stroke volume (ml/min)	% Recovery
1	38	16	54	221.04	60.77	125	82.18	15.07	0.19	12.67	-13.42	0.244	21%
2	44	16	60	216.05	61.45	107.4	76.77	14.43	0.19	9.16	-9.45	0.277	27.27%
3	42	18	60	252.65	58.84	112.27	76.65	15.11	0.152	10.59	-10.82	0.237	28.57%
4	42	18	60	260.54	63.45	115.33	80.75	15.516	0.14	11.22	-10.13	0.23	38.10%
5	42	18	60	269.19	56.08	111	74.42	14.97	0.13	10.15	-9.98	0.223	38.10%
6	42	18	60	188	52.56	114.53	73.22	15.28	0.224	10.33	-11.46	0.32	42.86%
7	36	16	52	256.65	62.96	98.26	74.73	11.46	0.16	8.85	-7.82	0.203	44.44%
8	40	20	60	251.02	58.06	111.58	74.91	15	0.153	9.14	-10.44	0.24	45%
9	44	16	60	218.7	57	129	80	17.13	0.183	12.67	-13.82	0.274	50%
AVE	41.1	17.3	58.4	237.1	59.0	113.8	77.1	14.9	0.2	10.5	-10.8	0.2	37.25%
Non-IPC ( failed )													
Sample	AO (ml/min)	CF (ml/min)	CO (ml/min)	Heart rate (beats /min)	Diastole (mmHg)	PSP (mmHg)	Mean BP (mmHg)	Total work (mW)	Ejection fraction	dP/dTmax	dP/dTmin	Stroke volume (ml/min)	% Recovery
10	44	20	64	303.75	57.19	108.67	74.46	15.67	0.14	10.36	-9.02	0.21	0.00%
11	38	16	54	264.98	55.75	109	73.5	13.07	0.14	10.54	-10.26	0.204	0.00%
12	48	18	66	275.24	55.945	109.31	70.4	13.273	0.12	12.53	-11.57	0.24	0.00%
13	40	18	58	278.6	53.48	113.16	73.64	14.82	0.12	11.26	-11.82	0.21	0.00%
14	48	16	66	289.42	55.9	114.3	71.9	14.9	0.12	10.86	-11.43	0.22	0.00%
15	44	16	60	257.47	58.5	109.9	75.09	14.71	0.166	10.94	-12.03	0.233	0.00%
16	42	18	60	240.31	58.93	107	74.89	14.39	0.096	7.71	-11.01	0.253	0.00%
17	48	20	68	303.11	51.78	114	72.49	17.52	0.105	12.29	-12.29	0.224	0.00%
18	44	18	62	282.6	57.45	110.11	75	15.4	0.13	11.37	-10.77	0.22	0.00%
19	40	20	60	264.37	60.72	104.9	75.45	14.145	0.14	8.81	-9.36	0.23	0.00%
20	44	16	60	248.11	57	110.36	74.78	14.86	0.14	9.79	-10.94	0.24	0.00%
AVE	43.64	17.82	61.45	272.72	56.56	109.80	73.98	15.17	0.13	10.59	-10.95	0.23	0.00%

Table 3.1 The haemodynamic parameters of the isolated rat hearts were measured after 15 min working heart before the period of sustained ischaemia, indicated by [red box]. The % recovery after 30 min reperfusion is indicated by [red box].

### **3.3) Haemodynamic parameters of the isolated rat hearts before sustained ischaemia (Table 3.1)**

The haemodynamic parameters of the isolated rat hearts after 15 min working heart, before the period of sustained ischaemia are indicated in table 3.1. The data presented in Table 3.1 indicates that there was no statistical differences between the parameters of the different animals before the start of the experiment, which could influence functional recovery of the ischaemic hearts. The percentage recovery (%) of the IPC hearts indicates functional recovery at the end of 30 min reperfusion, whilst the non-IPC hearts failed.

#### **3.3.1 The effects of IPC on the activation of the RISK pathway (PKB/Akt and ERK) during reperfusion (Figures 3.3.1 a-c).**

Results from the present study demonstrated that PKB/Akt was significantly activated in IPC compared to non-IPC ( $4.741 \pm 0.3414$  vs  $3.404 \pm 0.2574$ ;  $p < 0.05$ ) at 5 min reperfusion. At 15 min reperfusion, activation of PKB/Akt was significantly decreased in IPC compared to non-IPC ( $2.892 \pm 0.2638$  vs  $4.093 \pm 0.2086$ ;  $p < 0.05$ ). At 30 min reperfusion, activation of PKB/Akt was also significantly lower in IPC compared to non-IPC ( $1.501 \pm 0.07889$  vs  $3.550 \pm 0.2426$ ;  $p < 0.05$ ). ERKp44 was significantly increased in IPC compared to non-IPC ( $2.759 \pm 0.2107$  vs  $1.795 \pm 0.1207$ ;  $p < 0.05$ ) at 5 min reperfusion. ERKp42 was also significantly increased in IPC compared to non-IPC ( $1.485 \pm 0.1859$  vs  $0.9423 \pm 0.1112$ ;  $p < 0.05$ ) at 5 min reperfusion. However, at 30 min reperfusion, ERKp44 was significantly decreased in IPC compared to non-IPC ( $1.344 \pm 0.07658$  vs  $3.063 \pm 0.1312$ ;  $p < 0.05$ ). Similarly ERKp42 was also significantly attenuated in IPC compared to non-IPC ( $1.896 \pm 0.1168$  vs  $3.958 \pm 0.1678$ ;  $p < 0.05$ ).

#### **3.3.2 The effects of IPC on the activation of the stress kinase, p38 MAPK, during reperfusion (Figures 3.3.2 a-b).**

Interestingly, the stress kinase p38 MAPK was significantly activated in IPC compared to non-IPC ( $5.421 \pm 0.4415$  vs  $2.342 \pm 0.3440$ ;  $p < 0.05$ ) at 5 min

reperfusion. Similarly, p38 MAPK was also significantly increased in IPC compared to non-IPC ( $7.140 \pm 0.6138$  vs  $5.010 \pm 0.7842$ ;  $p < 0.05$ ) at 10 min reperfusion. At 15 min reperfusion, p38 MAPK was significantly attenuated in IPC compared to non-IPC ( $3.350 \pm 0.5955$  vs  $5.757 \pm 0.2600$ ;  $p < 0.05$ ). This was also the case at 30 min reperfusion, where p38 MAPK was significantly inhibited in IPC compared to non-IPC ( $1.015 \pm 0.05167$  vs  $2.662 \pm 0.1096$ ;  $p < 0.05$ ).

### **3.3.3 The effects of IPC on the activation of HSP-27 and HSP-70, substrates of p38 MAPK, during reperfusion (Figures 3.3.3 a-b).**

HSP-27, the downstream target of p38 MAPK was significantly activated in IPC ( $1.191 \pm 0.03369$  vs  $0.9598 \pm 0.0434$ ;  $p < 0.05$ ) compared to non-IPC at 5 min reperfusion. No significant differences were observed in HSP-70 between IPC and non-IPC at 10 min timepoint.

### **3.3.4 The effects of IPC on the activation of CREB and GSK-3 $\beta$ , substrates of PKB/Akt, during reperfusion (Figures 3.3.4 a-b).**

The transcription factor CREB, a downstream target of PKB/Akt, was significantly activated in IPC compared to non-IPC ( $1.592 \pm 0.1169$  vs  $1.062 \pm 0.05588$ ;  $p < 0.05$ ) at 5 min reperfusion as well as at 10 min reperfusion ( $2.483 \pm 0.2273$  vs  $1.558 \pm 0.2077$ ;  $p < 0.05$ ).

GSK-3 $\beta$ , another downstream target of PKB/Akt, was significantly activated in IPC compared to non-IPC ( $1.420 \pm 0.1909$  vs  $1.256 \pm 0.07675$ ;  $p < 0.05$ ) at 10 min reperfusion.

### **3.3.5 The effects of IPC on the activation of AMPK during reperfusion (Figure 3.3.5).**

In the present study AMPK was significantly activated in IPC ( $1.395 \pm 0.1303$ ;  $p < 0.05$ ) compared to non-IPC ( $1.141 \pm 0.04760$ ;  $p < 0.05$ ) at 5 min reperfusion. Similarly, AMPK was activated in IPC ( $1.697 \pm 0.4142$ ;  $p < 0.05$ ) compared to non-IPC ( $1.126 \pm 0.06978$ ;  $p < 0.05$ ).

### **3.3.6 The effects of IPC on the activation of CAMKII during reperfusion (Figure 3.3.6).**

No significant differences were observed in CAMKII activation at 5 and 10 minutes of reperfusion between IPC and non-IPC.

### **3.3.7 The effects of IPC on autophagy during reperfusion (Figures 3.3.7 a&b).**

The autophagy marker, **Beclin-1** was significantly activated in IPC compared to non-IPC ( $1.313 \pm 0.06631$  vs  $0.8700 \pm 0.04860$ ;  $p < 0.05$ ) at 5 min reperfusion. Another marker of autophagy, **LC3** was significantly activated in IPC compared to non-IPC ( $0.8019 \pm 0.05190$  vs  $0.6487 \pm 0.04898$ ;  $p < 0.05$ ) at 5 min reperfusion. At 10 min reperfusion however, LC3 was attenuated in IPC compared to non-IPC ( $1.293 \pm 0.08390$  vs  $1.778 \pm 0.09305$ ;  $p < 0.05$ ). Interestingly, at 30 min reperfusion LC3 activation was again significantly increased in IPC ( $0.8531 \pm 0.04587$  vs  $0.6065 \pm 0.08621$ ;  $p < 0.05$ ) compared to non-IPC.

### **3.3.8 The effects of IPC on apoptosis during reperfusion (Figures 3.3.8 a&b).**

Activation of cleaved caspase-3 was significantly increased in IPC compared to non-IPC ( $0.8531 \pm 0.04587$  vs  $0.6065 \pm 0.08621$ ;  $p < 0.05$ ) at 30 min reperfusion. No significant differences were observed in cleaved PARP at 30 minutes between IPC hearts and non-IPC hearts.

### 3.3) Results

#### 3.3.1 Investigating the involvement of PKB/Akt expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

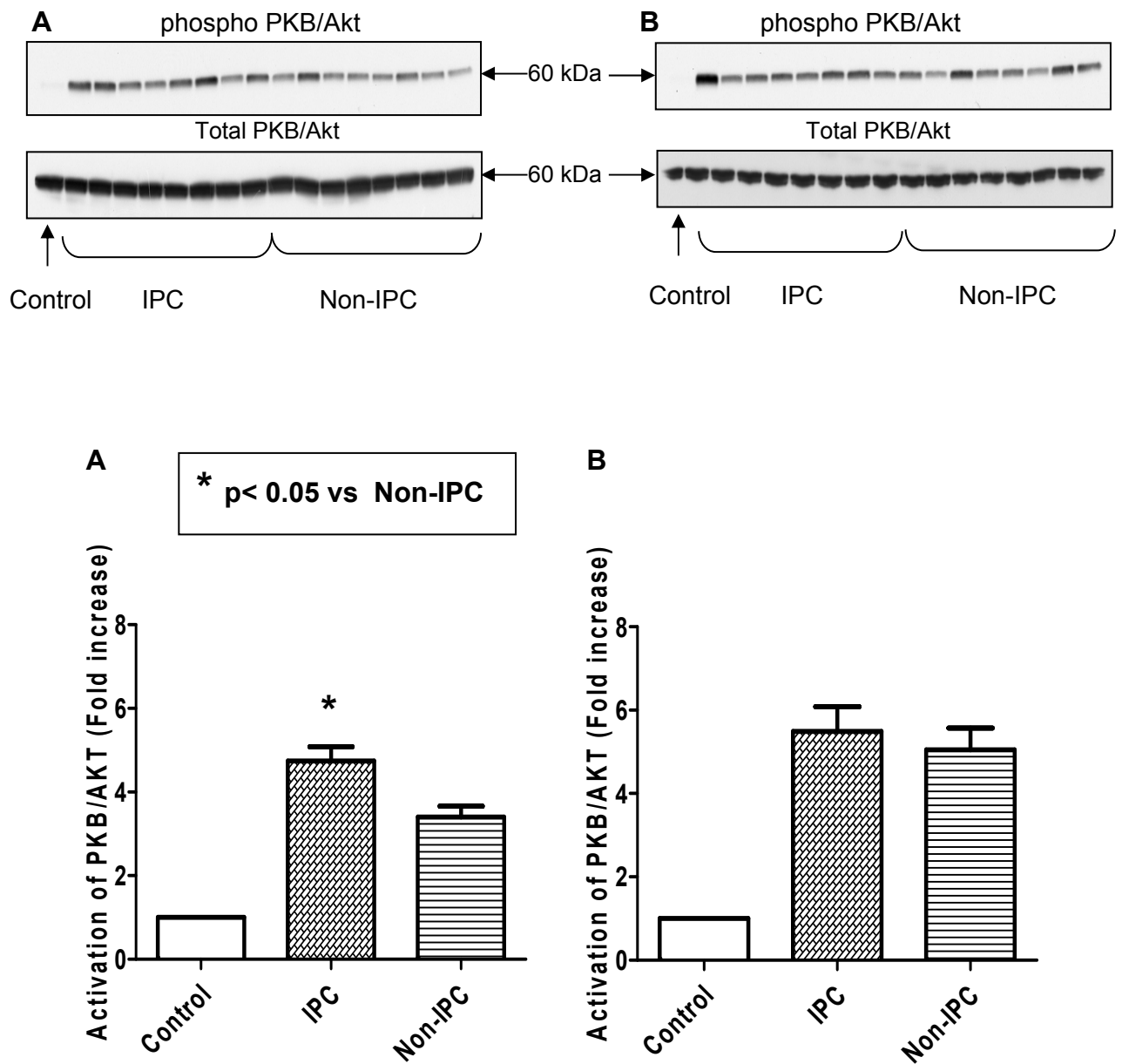


Figure 3.3.1(a) Activation of PKB/AKT during IPC and non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.1 Investigating the involvement of PKB/AKT expression and phosphorylation in IPC after 15 min (C) and 30 min (D) reperfusion.

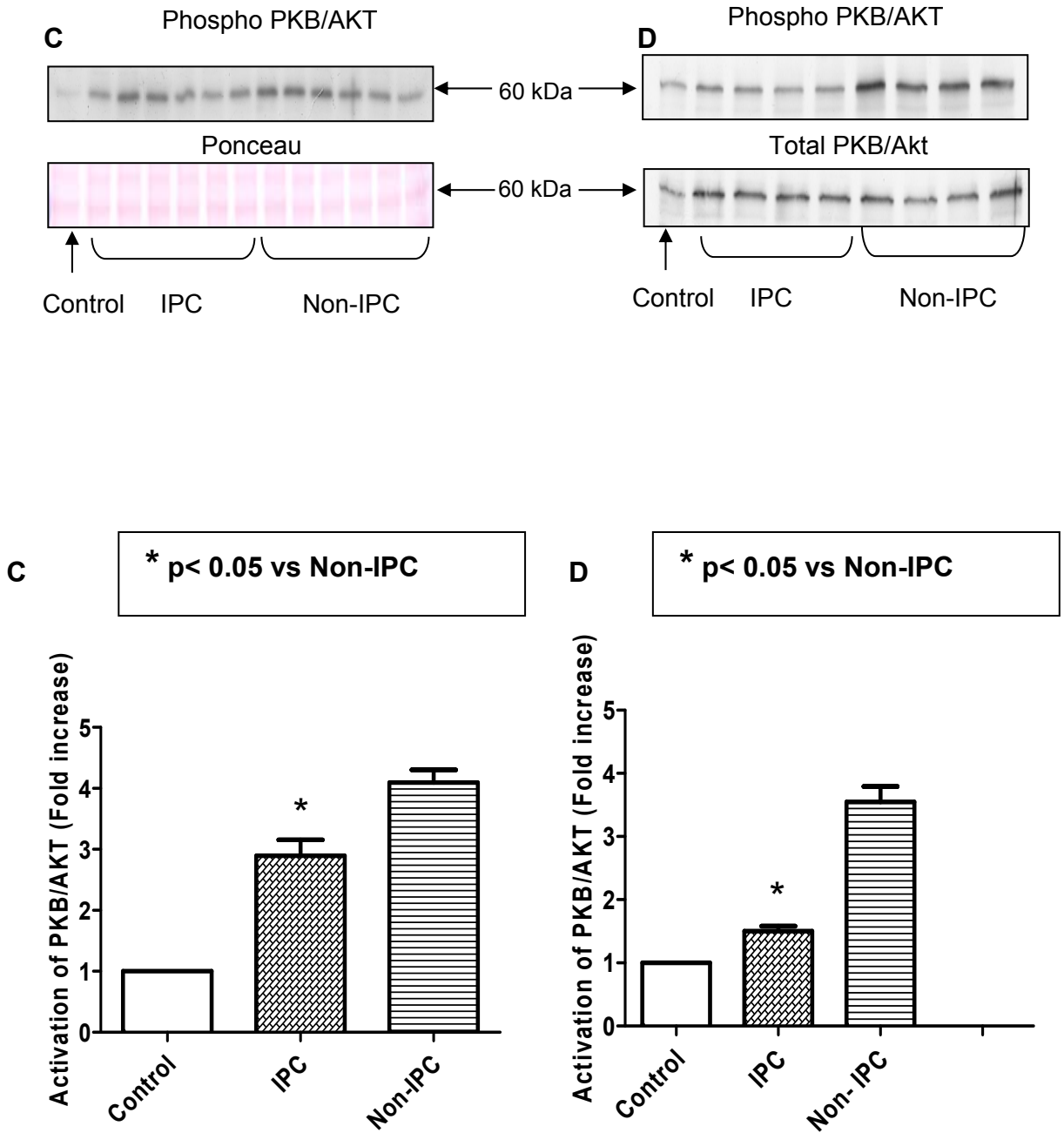


Figure 3.3.1(b) Activation of PKB/Akt during IPC and non-IPC at 15 min (C) and 30 min (D) reperfusion.

### 3.3.1 Investigating the involvement of ERK (p44/p42) MAPK expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

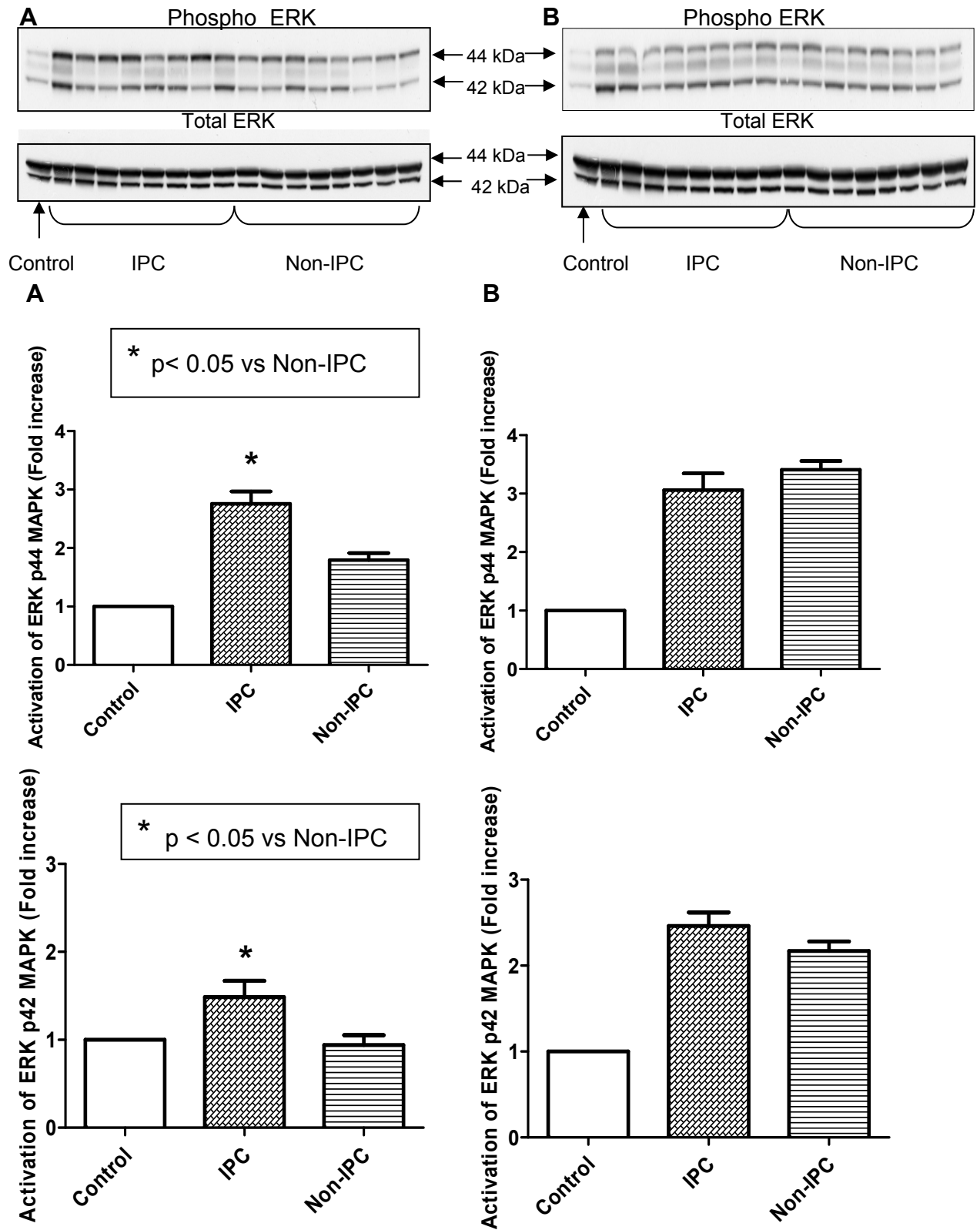


Figure 3.3.1(c) Activation of ERK (p44/p42) MAPK during IPC and non-IPC after 5 min (A) and 10 min (B) reperfusion.

### 3.3.1 Investigating the involvement of ERK (p44/p42) MAPK expression and phosphorylation in IPC after 15 min (C) and 30 min (D) reperfusion.

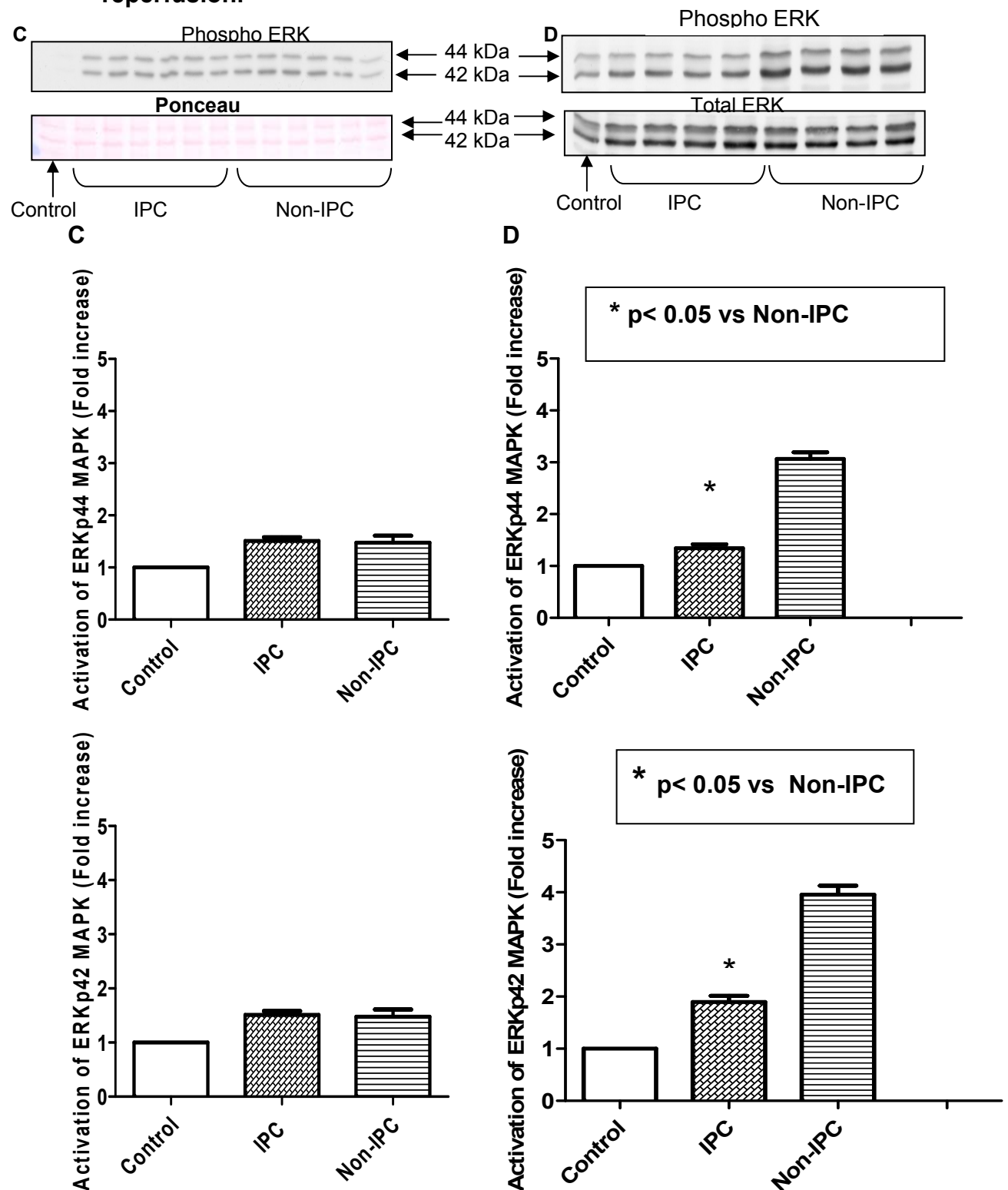


Figure 3.3.1(d) Activation of ERK (p44/p42) MAPK during IPC and non-IPC at 15 min (C) and 30 min (D) reperfusion.



### 3.3.2 Investigating the involvement of p38 MAPK expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

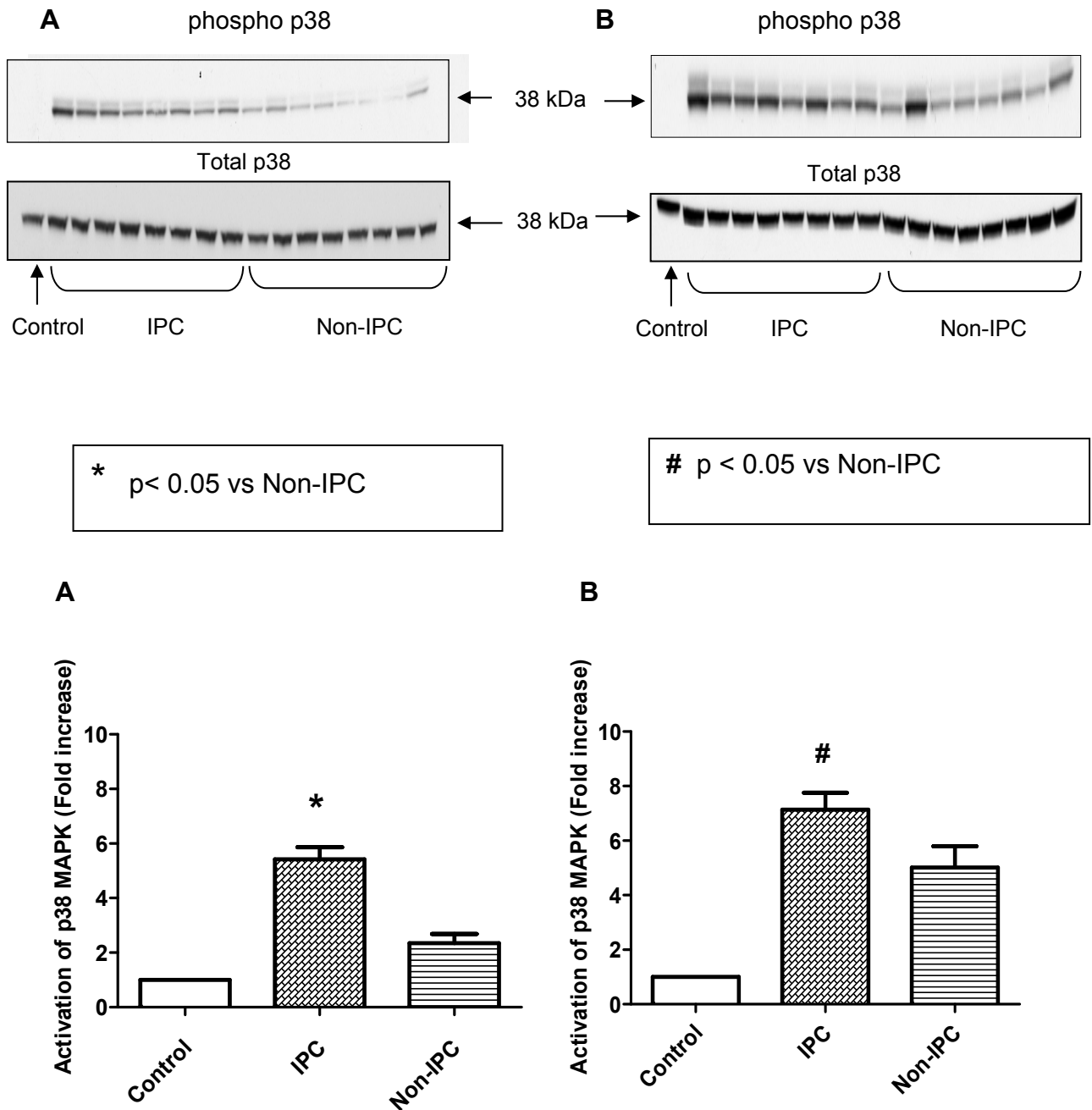


Figure 3.3.2(a)      Activation of p38 MAP Kinase during IPC and non-PC after 5 min (A) and 10 min (B) reperfusion.

### 3.3.2 Investigating the involvement of p38 MAPK expression and phosphorylation in IPC after 15 min (C) and 30 min (D) reperfusion.

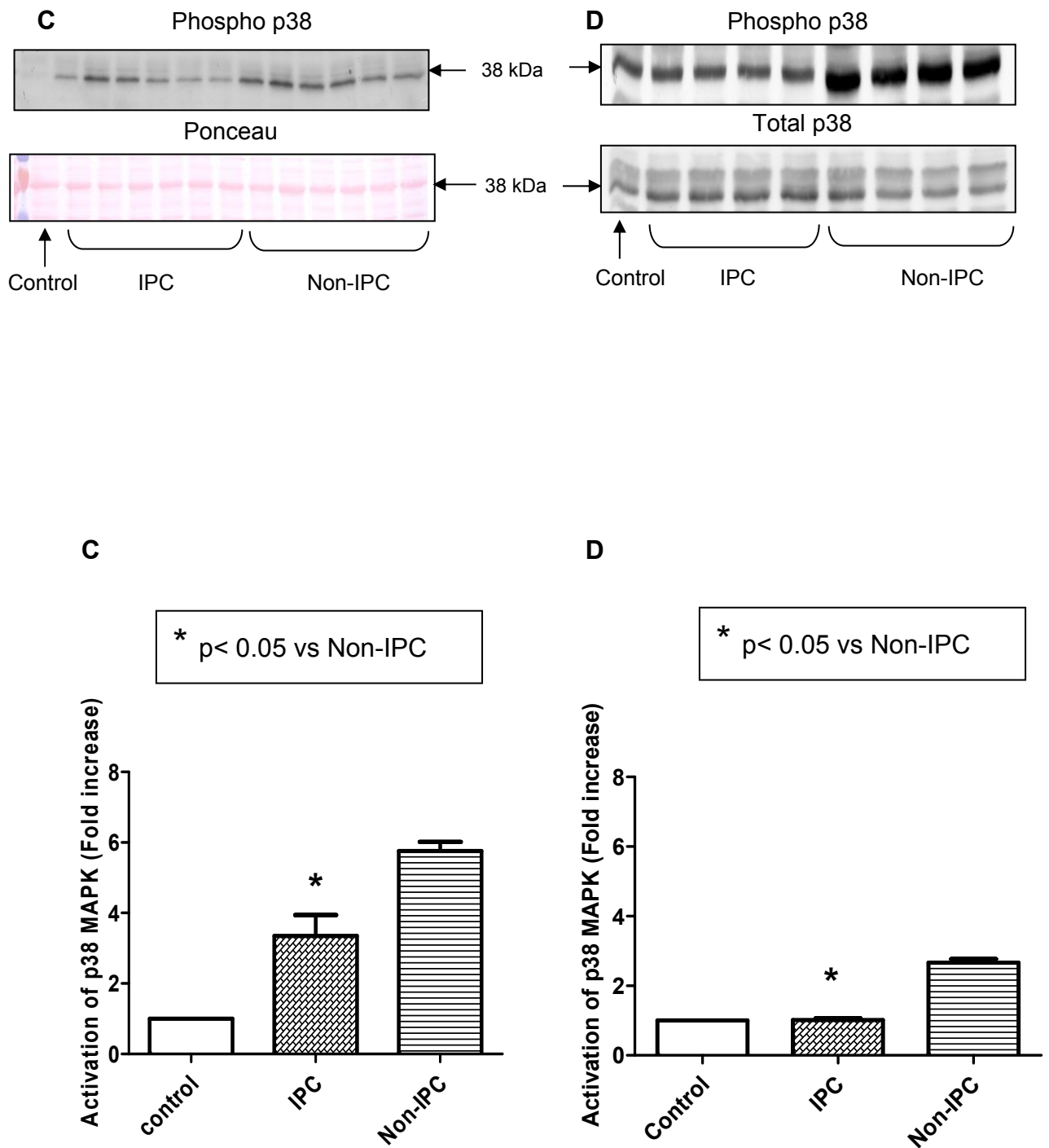


Figure 3.3.2(b) Activation of p38 MAPK during IPC and non-IPC at 15 min (C) and 30 min (D) reperfusion.

### 3.3.3 Investigating the involvement of HSP-27 expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

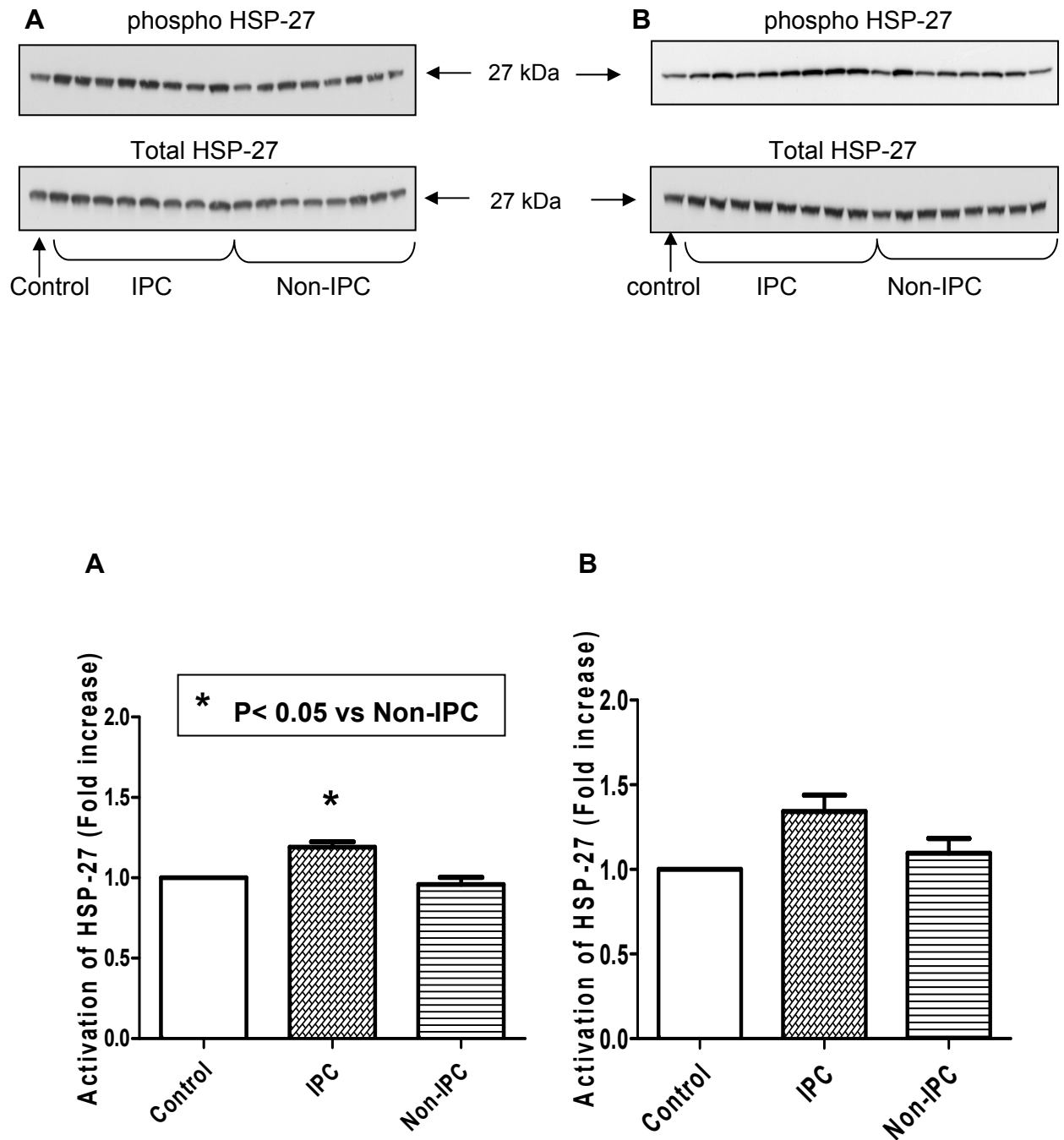


Figure 3.3.3(a) Activation of HSP-27 during IPC and non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.3 Investigating the involvement of HSP 70 expression in IP after 5 min (A) and 10 min (B) reperfusion.

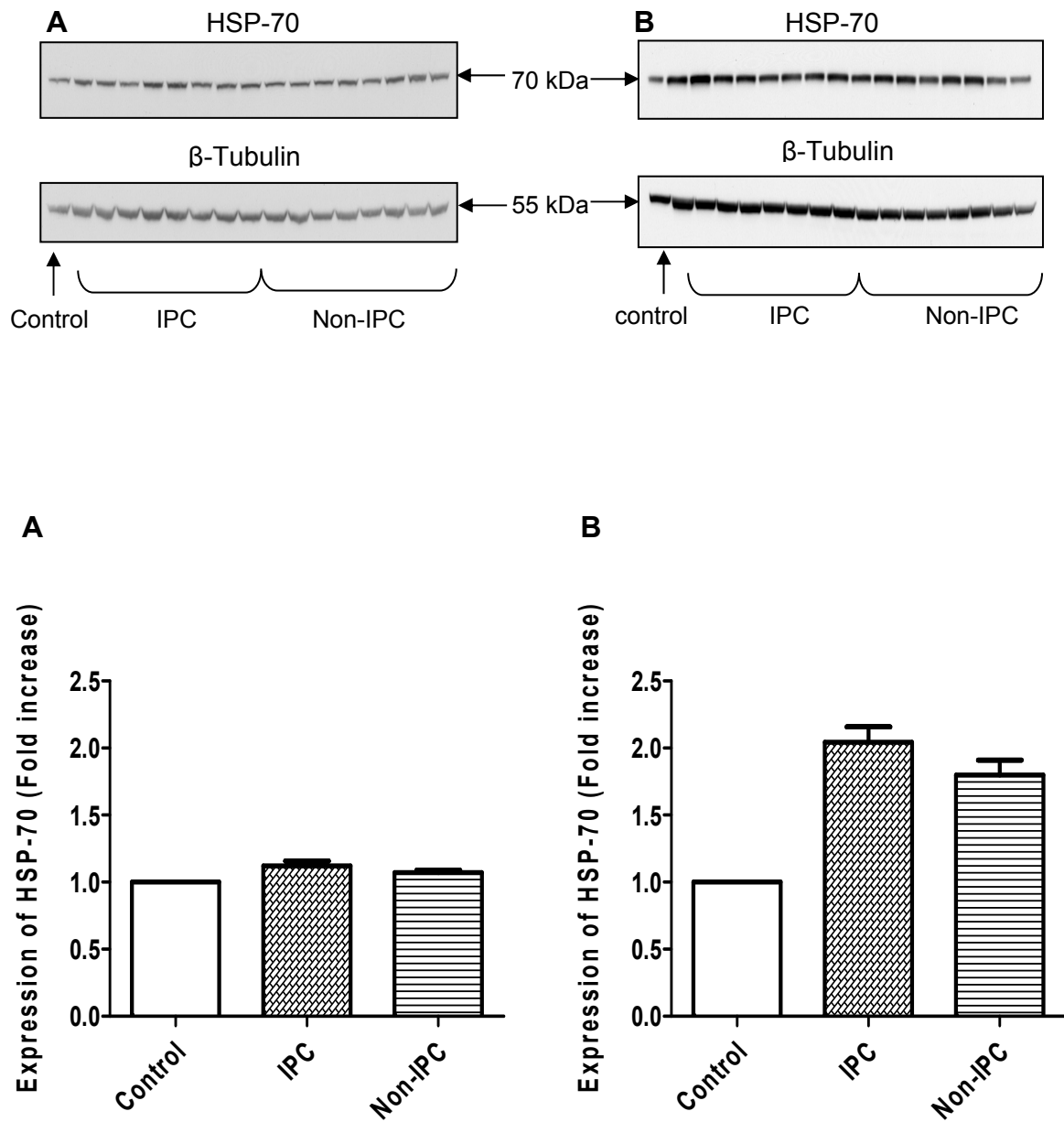


Figure 3.3.3(b) Expression of HSP-70 in ischaemic preconditioning (IPC) and non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.4 Investigating the involvement of CREB expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

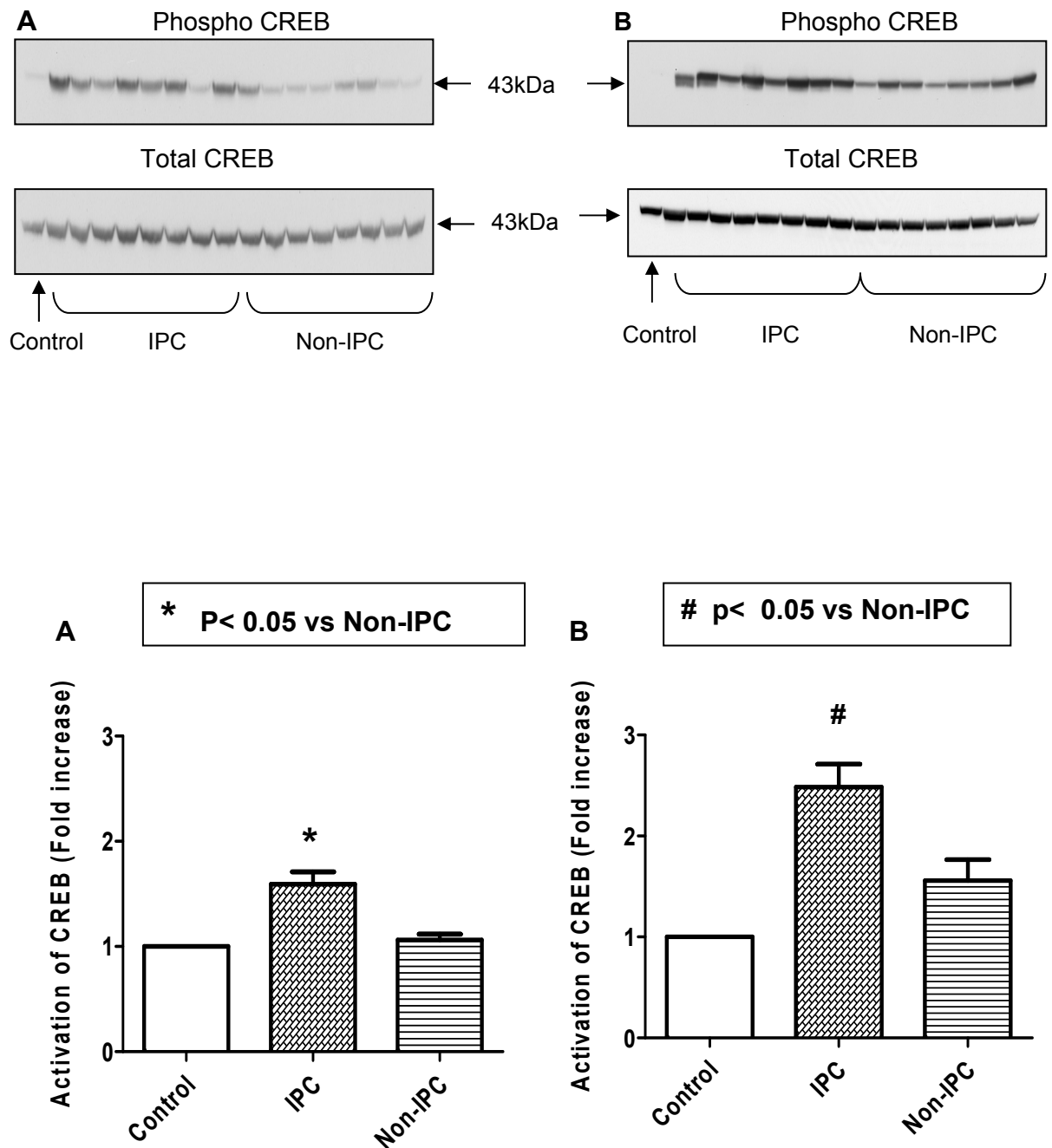


Figure 3.3.4(a) Activation of CREB during IPC and non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.4 Investigating the involvement of GSK-3 $\beta$ expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

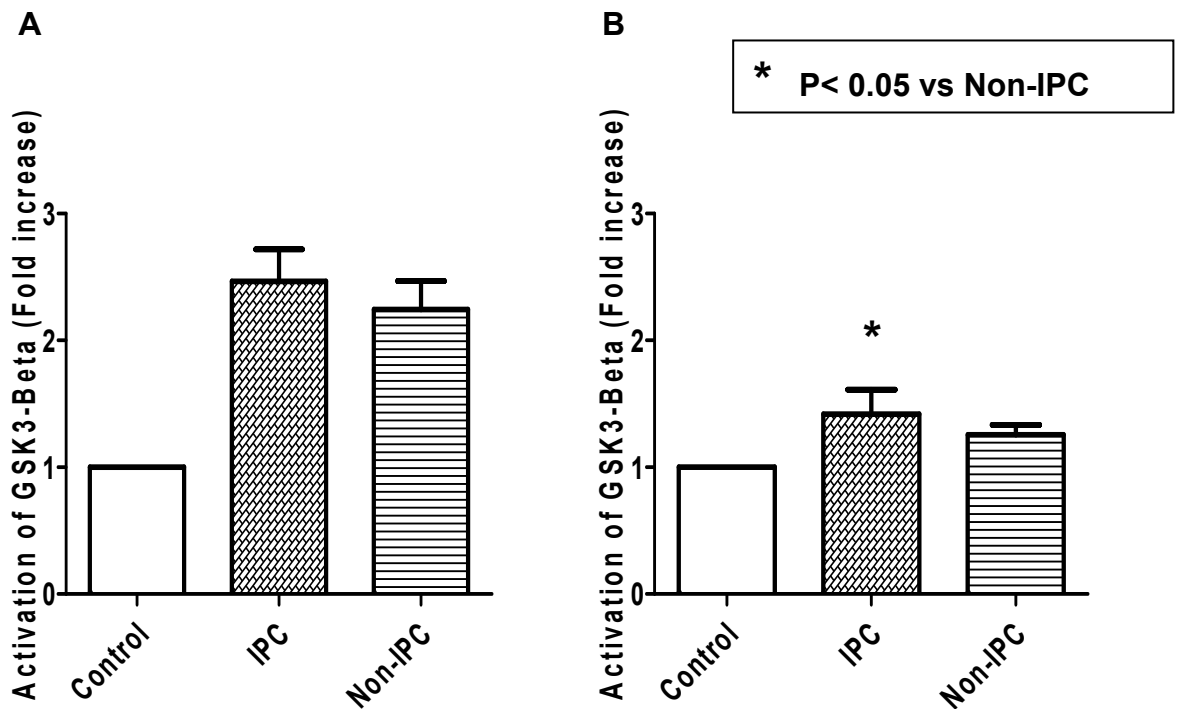
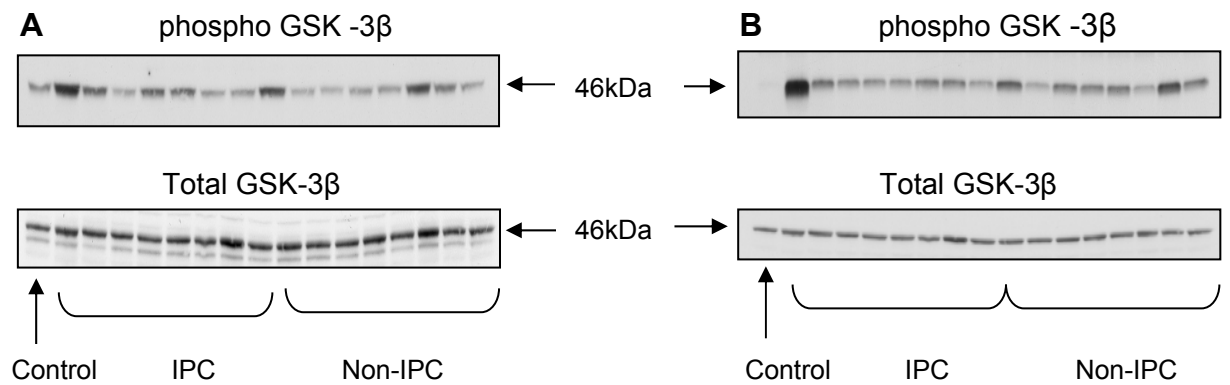


Figure 3.3.4(b) Activation of GSK3- $\beta$  in IPC and non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.5 Investigating the involvement of AMPK expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.

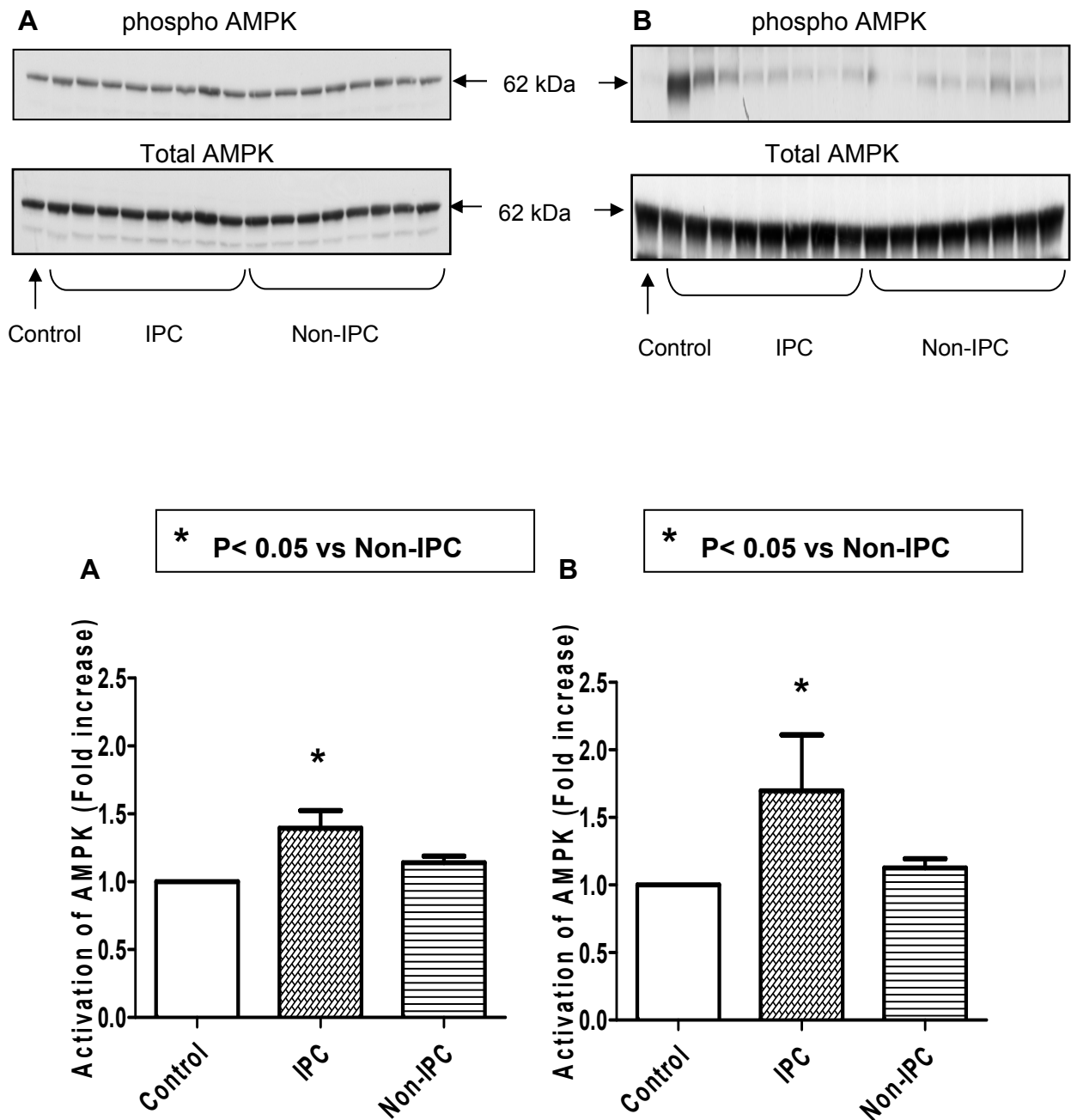
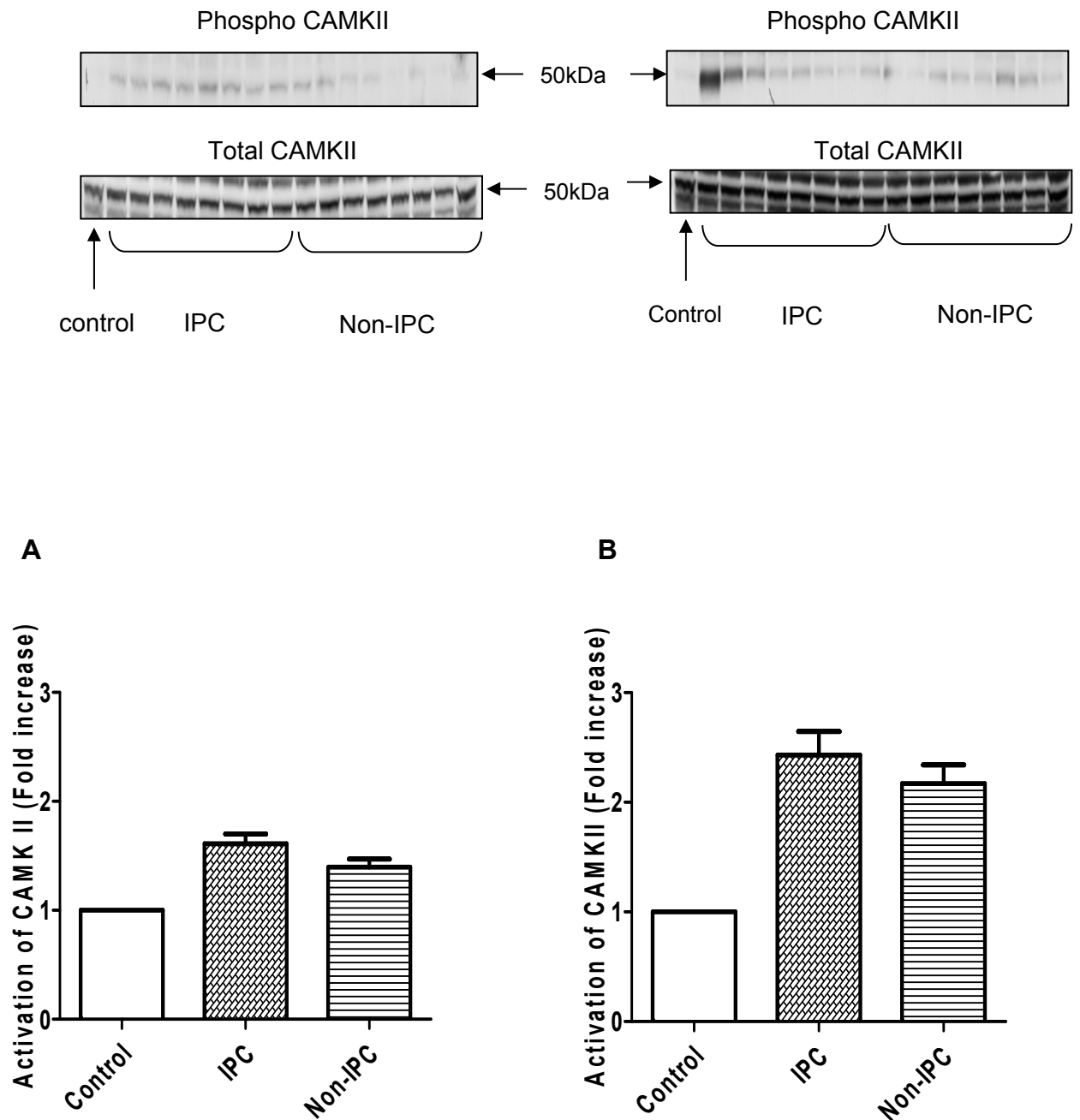


Figure 3.3.5 Activation of AMP kinase during IPC and Non-IPC at 5 min (A) and 10 min (B) reperfusion.

### 3.3.6 Investigating the involvement of CAMKII expression and phosphorylation in IPC after 5 min (A) and 10 min (B) reperfusion.



**Figure 3.3.6** Activation of CAMKII in IPC and non-IPC at 5 min (A) and 10 min (B) reperfusion.



### 3.3.7 Investigating the involvement of Beclin-1 activation in IPC after 5 min (A), 10 min (B) and 30 min (C) reperfusion.

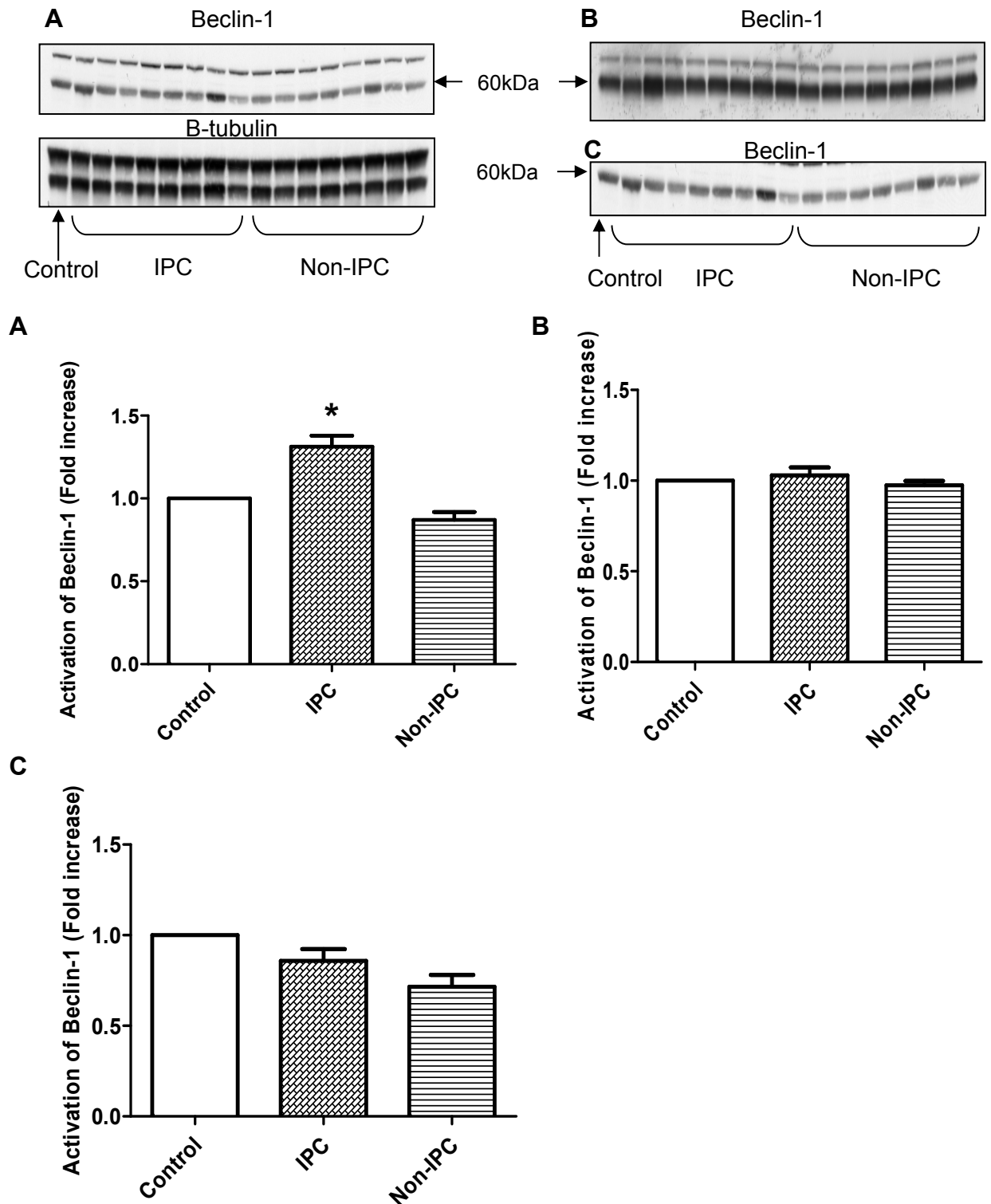


Figure 3.3.7(a) Activation of Beclin-1 in IPC and non-IPC at 5 min (A), 10 min (B) and 30 min (C) reperfusion.

### 3.3.7 Investigating the involvement of LC-3 activation in IPC after 5 min

(A), 10 min (B) and 30 min (C) reperfusion.

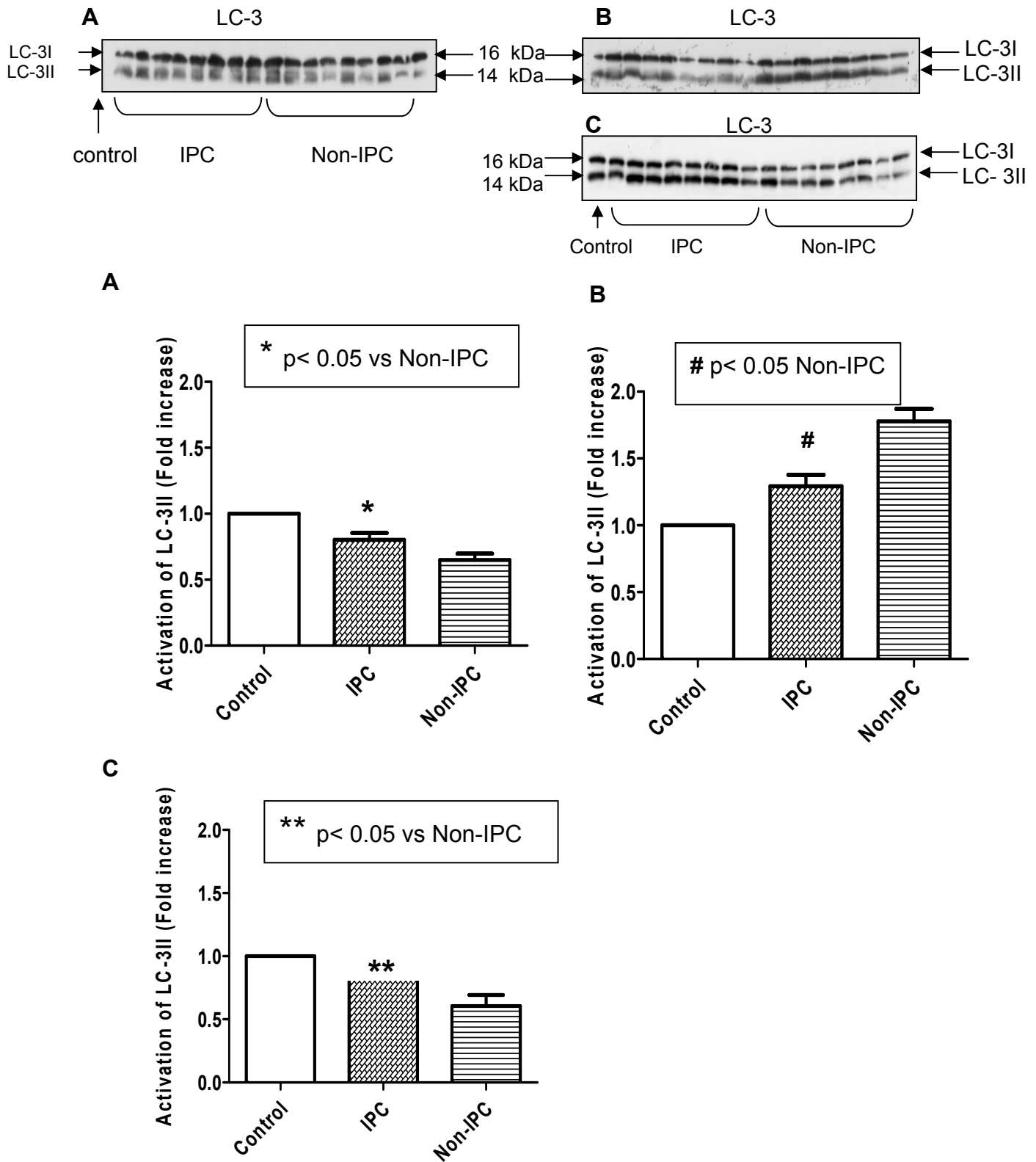
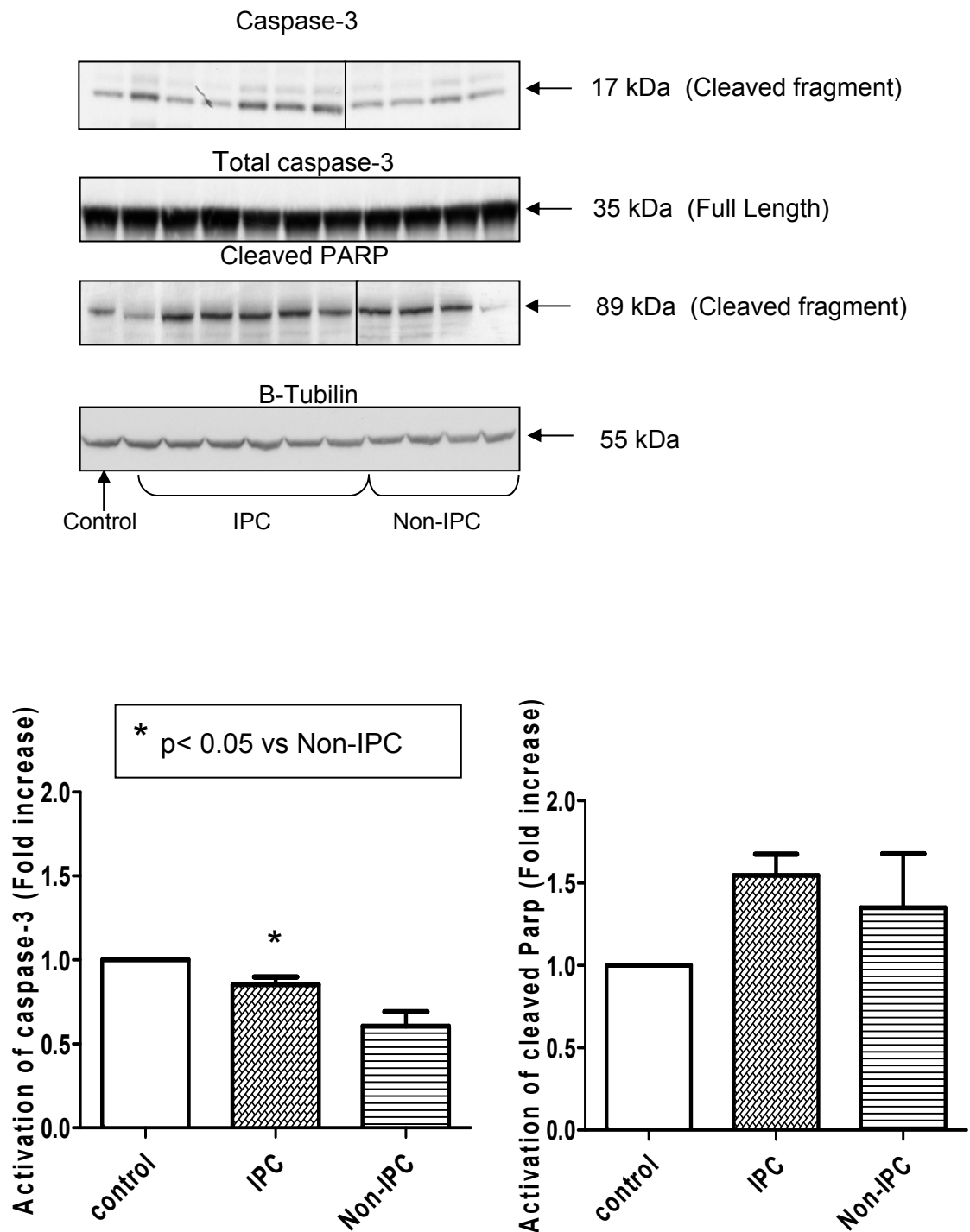


Figure 3.3.7(b) Activation of LC-3 in ischaemic preconditioning (IPC) and non-IPC at 5 min (A), 10 min (B) and 30 min (C) reperfusion.

### 3.3.8 Investigating the involvement of caspase-3 activation and PARP cleavage in IPC after 30 min reperfusion.



**Figure 3.3.8** Activation of caspase-3 and cleaved PARP in IPC and non-IPC at 30 min reperfusion.

### 3.4) Discussion

One of the most challenging aspects of research on signal transduction pathways is the fact that the chemical signals are usually very compartmentalized. For example, phospholipid-activated PKC is known to attach to docking sites where PKC phosphorylates its substrates only in the vicinity of the docking sites (Mochly-Rosen et al., 1987). Furthermore, a very few signals are distributed globally within the cell. Some signals like phosphorylation of PKB/Akt and ERK are very robust and easy to measure, while others like PKC are very difficult since more than 10 isoforms can be activated independently by lipid cofactors.

Another critical factor which should be considered is that several signaling pathways are activated simultaneously during the IPC protocol. The usual technique for identifying a critical step in the pathway is to inhibit that step and test whether the response has been eliminated. However, inhibitors are not always very specific and multiple signals may occur simultaneously, therefore results must be interpreted with caution.

Another problem that confront researchers, is to decide when to measure the signal. Some investigators make biochemical measurements several hours after reperfusion so that the infarct size can be measured in the same heart. However, Downey and co-workers (2008) is of the opinion that any signal at such a late time point in reperfusion would be irrelevant. They also suggested that triggers should obviously be measured during the trigger phase, while mediator signals should be measured in the first minutes of reperfusion. Furthermore, many researchers just measure a signal at a single time point during reperfusion and then assumes that activation/inhibition of the signals are associated with IPC-induced protection of the heart. Clearly, our understanding of events in the mediator phase is still limited; therefore there is a need for the characterization of the signaling mechanisms in the early reperfusion phase.

In light of the above mentioned, the aim of this part of the study was to determine the role of possible mediators during different time points in reperfusion after IPC and to compare it with non-IPC hearts at the same time points. These activation patterns of the kinases after IPC were summarized in Table 3.2.

**Table 3.2 A summary of the activation patterns of the kinases during reperfusion after IPC compared to Non-IPC hearts.**

Activation of the RISK pathway and p38 MAPK				
	PKB/Akt	ERK44	ERK42	p38 MAPK
Reperfusion				
5 min	↑	↑	↑	↑
10 min	↔	↔	↔	↑
15 min	↓	↔	↔	↓
30 min	↓	↓	↓	↓
Activation of the heat shock proteins				
	HSP-27	HSP-70		
Reperfusion				
5 min	↑	↔		
10 min	↔	↔		
Activation of PKB/Akt substrates and AMPK				
	CREB	GSK-3β	AMPK	
Reperfusion				
5 min	↑	↔	↑	
10 min	↑	↑	↑	
Activation of autophagy and apoptosis				
	LC-3	Beclin-1	Caspase-3	PARP
Reperfusion				
5 min	↑	↑		
10 min	↓	↔		
30 min	↑	↔	↑	↔

### 3.4.1 The RISK pathway and PKB/Akt substrates

A significant increase in PKB/Akt was observed after 5 minutes reperfusion in the IPC hearts compared to the non-IPC hearts. This was however, a transient activation where no significant differences were observed at 10 minutes and where the signal was attenuated at 15 and 30 minutes reperfusion compared to non-IPC hearts. ERK44 and ERK42 were also significantly increased at 5 minutes reperfusion, while no significant differences were observed at 10 and 15 minutes. A significant decrease in ERK44 and ERK42 was also observed at the 30 minute time point compared to the non-IPC hearts. Our results are in accordance with the literature where activation of PKB/Akt and ERK1/2 conferred powerful cardioprotection when activated early in reperfusion [Yellon & Baxter, 1999; Housenloy & Yellon, 2004].

PKB/Akt plays a dual role in the mechanisms of protection during IPC - during the **triggering phase** of protection (where receptor activation prepares cardiomyocytes for subsequent insults) and in the **mediator phase** (where protective mechanisms suppress concurrent ischaemia and/or reperfusion injury) [Downey et al., 2007; Cao et al., 2008]. In fact, the infarct size-limiting effects of IPC is abolished by inhibition of PI3-Kinase either during IPC (before ischaemia) or at the time of reperfusion. During the trigger phase of IPC, it is possible that cytosolic PKB/Akt plays a role in protection as a series of studies by Downey and others demonstrated that activated PKB/Akt transmitted a signal from PI3-K to ERK with eNOS and PKG as downstream substrates, which ultimately activates the mitochondrial ATP-sensitive potassium channels (mK<sub>ATP</sub> channels) [Housenloy et al., 2005; Downey et al., 2007; Cohen et al., 2007]. The protective role of PKB/Akt in the mediator phase of IPC may involve the mitochondria, as phosphorylation of PKB/Akt during early reperfusion induces mitochondrial translocation of hexokinase [Zuurbier et al., 2005], a PKB/Akt substrate, and also inhibits opening of the mPTPs upon reperfusion [Housenloy et al., 2002; Moon et al., 2006].

A substrate of PKB/Akt, GSK-3 $\beta$ , showed increased phosphorylation at 10 minutes of reperfusion after IPC compared to non-IPC hearts. Numerous studies showed that **phosphorylation at Ser9 of GSK-3 $\beta$** , which **results in its**

**inhibition**, is a key event in signaling mechanisms during IPC [Tong et al., 2002; Gross et al., 2004]. Nishihara and co-workers (2006) found a close negative correlation between tissue levels of phospho-GSK-3 $\beta$  at 5 minutes after reperfusion and infarct size after 2 h reperfusion in rat hearts *in vivo*. Interestingly, Nishihara, *et al* (2007) has also demonstrated that GSK-3 $\beta$  translocates to the mitochondria and physically interacts with adenine nucleotide translocase (ANT) and the voltage-dependent anion channel (VDAC), mPTP subunits, after ischaemia/reperfusion. A significant correlation between the thresholds for mPTP opening in isolated cardiac mitochondria and the ratio of phospho-GSK-3 $\beta$  to total GSK-3 $\beta$  was found in mitochondrial samples of the heart [Miki et al., 2009]. It is thus suggested that inactivation of GSK-3 $\beta$  through PKB/Akt phosphorylation increases the threshold of mPTP opening and tolerance to ischaemia/reperfusion injury [Miura et al., 2010]. Although GSK-3 was initially discovered and named for its role in regulating glycogen metabolism, Omar and co-workers (2010) was the first to link this important effect on myocardial metabolism with cardioprotection. Their research highlighted the ability of GSK-3 to regulate myocardial glycogen and glucose metabolism and demonstrated an additional mechanism linking GSK-3 inhibition to enhanced recovery of post-ischaemic mechanical function. Inhibition of GSK-3 during reperfusion stimulated glycogen synthesis which repartitions glucose-6-phosphate away from the glycolytic pathway. The reduced rate of glycolysis lessens H<sup>+</sup> production from glucose metabolism and subsequently reduces Ca<sup>2+</sup> overload. These effects limit left ventricular dysfunction in early reperfusion and may contribute to improvements in mitochondrial function and cell viability.

IPC also induces a significant increase in cyclic AMP (cAMP)-response element binding protein (CREB) phosphorylation during 5 and 10 minutes of reperfusion when compared to non-IPC hearts. PKB/Akt has been shown in some cell types to increase the expression of the antiapoptotic gene, *bcl-2* [Skorski et al., 1997; Pugazhenthii et al., 2000]. Induction of *bcl-2* promoter activity by IGF-1 was shown to occur via a PKB/Akt pathway involving CREB. CREB is a direct target for phosphorylation by PKB/Akt [Du & Montminy, 1998] and this phosphorylation occurs on a site that increases binding of CREB to accessory proteins necessary for induction of genes containing cAMP response elements (CREs) in their

promoter regions. CREB has also been shown to mediate PKB/Akt-induced expression of another antiapoptotic gene, *mcl-1* [Wang et al., 1999].

### 3.4.2 p38 MAPK and Heat Shock Proteins

A significant increase in p38 MAPK activation occurred at 5 and 10 minutes reperfusion and was attenuated at 15 and 30 minutes of reperfusion after IPC when compared to non-IPC hearts. Although the majority of studies showed p38 MAPK activation during the triggering phase of IPC [Sato et al., 2000; Nakano et al., 2000; Fryer et al., 2001; Housenloy et al., 2005], the role of p38 MAPK as a potential mediator of protection in the preconditioned hearts remains controversial. The p38 $\alpha$ -MAPK isoform appears to be required during IPC-induced protection of the heart [Sicard et al., 2010]. Potential p38 MAPK-mediated protective mechanisms include the phosphorylation of small heat shock protein, Hsp-27, which stabilizes the actin cytoskeleton during ischaemia [Rouse et al., 1994; Li et al., 2008; Clements et al., 2011]. In addition, HSP-27 decreases the intracellular ROS content and prevent apoptotic cell death, by binding directly to cytochrome c [Paul et al., 2002; Samali et al., 2001].

### 3.4.3 CAMKII

In the present study no differences in CAMKII activation and expression were observed between IPC and Non-IPC at 5 and 10 minutes reperfusion. This is an unexpected finding as attenuated activation of CAMKII was shown to be cardioprotective [Jingdong et al., 2007]. Since the anti-apoptotic RISK pathway is activated in IPC at 5 minutes reperfusion, one would expect CAMKII to be attenuated in IPC at this timepoint. Since CAMKII is a pro-apoptotic signaling molecule these results suggest that at 10 minutes reperfusion the IPC and Non-IPC hearts are equally protected against Ischaemia/reperfusion injury. This statement can be confirmed by the finding that the anti-apoptotic RISK pathway is attenuated in IPC at 10 min reperfusion. These results suggest that IPC-induced protection is lost after 5 minutes reperfusion.

Calcium/Calmodulin-dependent protein kinase II (CAMKII) is a multifunctional signaling molecule in the heart and activated in response to  $\beta$ -adrenergic



stimulation [Anderson 2009]. Excessive catecholamine exposure and subsequent  $\beta$ -stimulation increases the activation and expression of CAMKII in the heart, leading to the expression of hypertrophic genes. CAMKII therefore participates in the transition from early structural heart disease to heart failure and sudden death [Anderson 2009]. CAMKII is also activated in response to myocardial infarction, heart failure, ischaemia reperfusion injury and myocardial cell death [Mei-ling et al., 2012]. Inhibition of CAMKII in cardiomyocytes prevented the maladaptive remodeling after myocardial infarction [Zhang et al., 2005; Yang et al., 2006; Wu et al., 2006]. Inhibition of CAMKII in Langendorff perfused mouse hearts were resistant to ischaemia/reperfusion injury [Li et al., 2006].

#### **3.4.4 AMPK, the energy sensor of the cell**

A significant increase in AMP-activated protein kinase (AMPK) was observed at 5 and 10 minutes of reperfusion after IPC compared to non-IPC hearts. The energy reserve of the heart is rapidly depleted during ischaemia [Nishida et al., 2009]. AMPK act as a sensor for energy deprivation, and activation of AMPK mediates metabolic adaptation during ischaemia. AMPK plays an important role in mediating ischaemia-induced autophagy in cardiac myocytes [Zhang et al., 2013]. The phosphatidylinositol kinase homolog of the mammalian target of rapamycin (mTOR), is a sensor of the nutrient status in the cell and is an important negative regulator of autophagy [Meijer & Codogno, 2004]. AMPK has been shown to inhibit mTOR and thereby induces protective autophagy [Papandreou et al., 2008].

#### **3.4.5 Autophagy**

LC-3II and Beclin-1, two markers of autophagy, were significantly upregulated at 5 minutes reperfusion after IPC compared to non-IPC hearts. Interestingly, at 10 minutes reperfusion, LC-3II was attenuated, but upregulated again at 30 minutes. No differences in Beclin-1 were observed at 10 and 30 minutes reperfusion. Autophagy can be detected under basal conditions in the heart and the level increases during ischaemia. It is generally accepted that activation of autophagy during ischaemia is protective and important for cell survival and maintenance of cardiac function [Decker & Wildenthal, 1980; Matsui et al., 2007]. The level of

autophagy further increases during reperfusion and the production of ROS was suggested to be the main inducer of autophagy during reperfusion. However, activation of autophagy during reperfusion has been shown to be detrimental to the heart when it is associated with activation of the autophagy marker Beclin-1. [Zhang et al., 1013].

It has been shown recently that autophagy is activated during IPC [Yitzhaki et al., 2009; Yan et al., 2009]. This is in agreement with our results where we have observed a significant increase in the activation of both Beclin-1 and LC-3II at 5 min reperfusion.

### **3.4.6 Apoptosis**

A significant increase in cleaved caspase-3 was observed at 30 min reperfusion after IPC compared with the non-IPC hearts. These results are not in agreement with the literature, since it is proposed that IPC has anti-apoptotic effects. However, in the present study the RISK pathway which has anti-apoptotic effects was attenuated in IPC at 30 min reperfusion compared to non-IPC. These results may suggest that when the Risk pathway is attenuated, protection is lost and cells are committed to undergo apoptosis. Recent studies have demonstrated that the release of cytochrome c from the mitochondria leads to the activation of caspase-3 [Zou et al., 1997]. In normal cells, cytochrome c is confined to the mitochondria with no activation of caspase-3 [Narula et al., 1999]. The activation of caspase-3 usually leads to cleavage of its cytoplasmic substrate PKC- $\delta$  and cleavage of its nuclear substrate PARP for the induction of apoptosis. PARP cleavage however, is not always demonstrated in cardiomyopathic hearts, suggesting a mechanism that links mitochondrial release of cytochrome c to activation of caspase-3. Cytochrome c release and activation of caspase-3 occur both in ischaemic and idiopathic dilated cardiomyopathic hearts in a similar way. [Narula et al., 1999]. Although cytochrome c release from mitochondria, accompanied by caspase activation are almost generally observed during apoptosis, the ultrastructural alterations of apoptosis in nuclei as a result of PARP cleavage is not always observed [Narula et al., 1999]. Activation of caspase 3 in the absence of PARP cleavage as observed in the present study, is therefore in agreement with the literature.

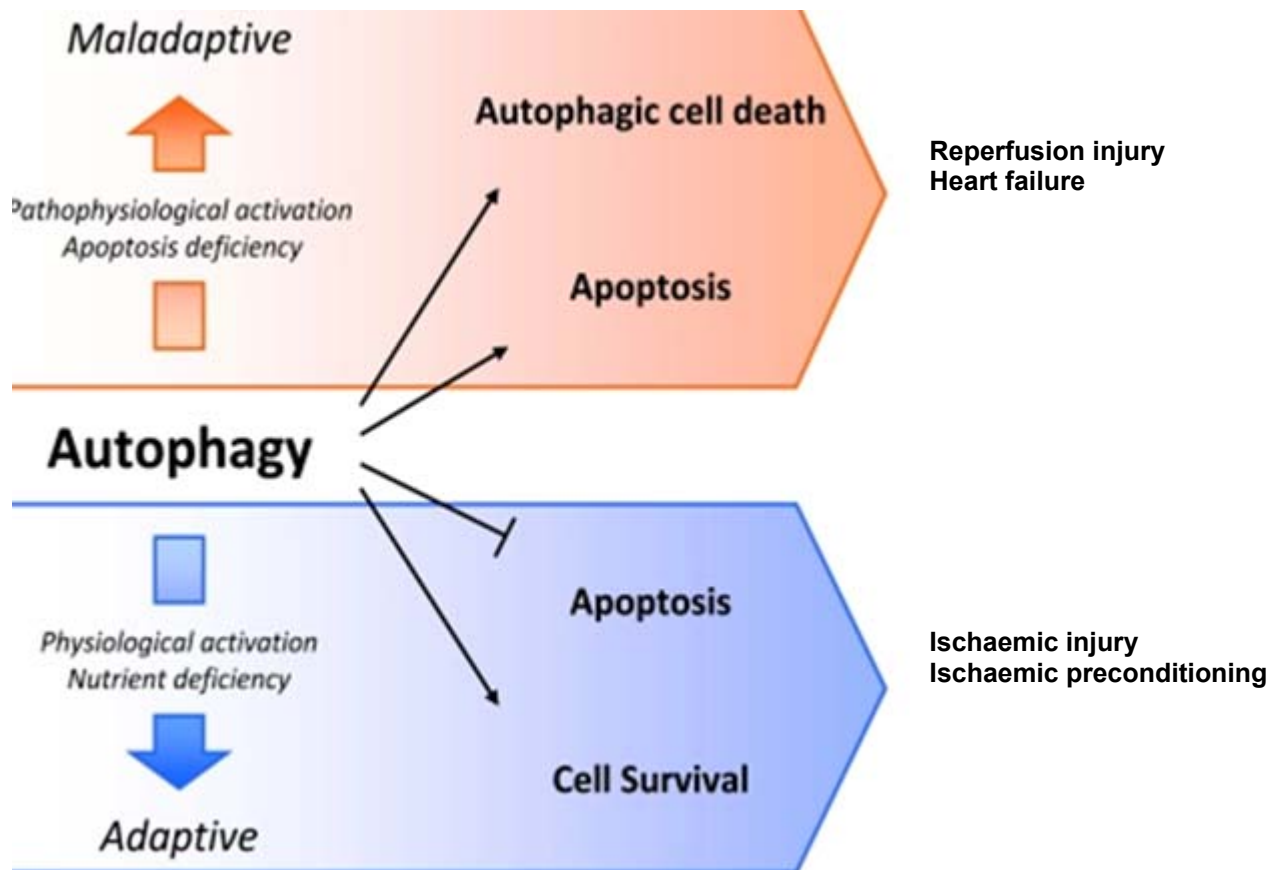


Figure 3.4: The double edge sword of autophagy (modified from Ryter et al., 2010)

- **Beneficial role of autophagy:** homeostatic turnover of damaged organelles and protein, promoting recycling of metabolic building blocks for the generation of energy.
- **Excessive autophagy** associated with aberrant degradation of intracellular constituents, leading to type II (autophagic) cell death.
- Relationship between **autophagy and apoptosis** is incompletely understood – both impaired and excessive autophagy can lead to apoptosis.

### 3.5) Conclusion

The aim of this study was to identify possible mediators of IPC during different timepoints of reperfusion. The activation patterns of the kinases (Table 3.2), suggest that the RISK pathway (PKB/Akt and ERK MAPK) as well as p38 MAPK may act as possible mediators of IPC. As already mentioned, some investigators measure kinase activation several hours after reperfusion so that infarct size can be measured in the same heart. Our results however, demonstrated that activation of the RISK pathway is an early event in IPC. Interestingly, p38 MAPK activation in IPC at 5 minutes reperfusion was accompanied by activation of the RISK pathway. This is an unexpected finding and to our knowledge has never been reported before in the setting of IPC and ischaemia/reperfusion damage. In addition this was accompanied by activation of HSP-27, CREB, AMPK, LC-3II and Beclin-1. These results suggest that activation of p38 MAPK in IPC may be potentially beneficial during early reperfusion.

According to Marais and co-workers 2001, IPC-induced protection is associated with an attenuation of p38 MAPK activation and enhanced activation of HSP-27 during reperfusion in the isolated working rat heart model. In this particular study however, the RISK pathway was not investigated and no explanation was offered for their observation that HSP-27 activation was accompanied by an attenuation of p38 MAPK activation.

Hausenloy and co-workers (2003), utilizing the isolated perfused rat heart model demonstrated that IPC results in activation of the RISK pathway at 15 minutes reperfusion. In this particular study activation of p38 MAPK and HSP-27 were not investigated [Hausenloy et al., 2003]

Results demonstrated that autophagy plays an important role in early (5 min) and late (30 min) reperfusion, suggesting that it could act as a mediator and possible end-effector in IPC. Currently there is no consensus as to whether activation of autophagy during ischaemia/reperfusion represents a protective or detrimental function. It is generally expected however, that the functional role of autophagy during ischaemia represents a beneficial role, as it is normally associated with activation of the energy sensor of the cell, AMPK [Ryter et al.,

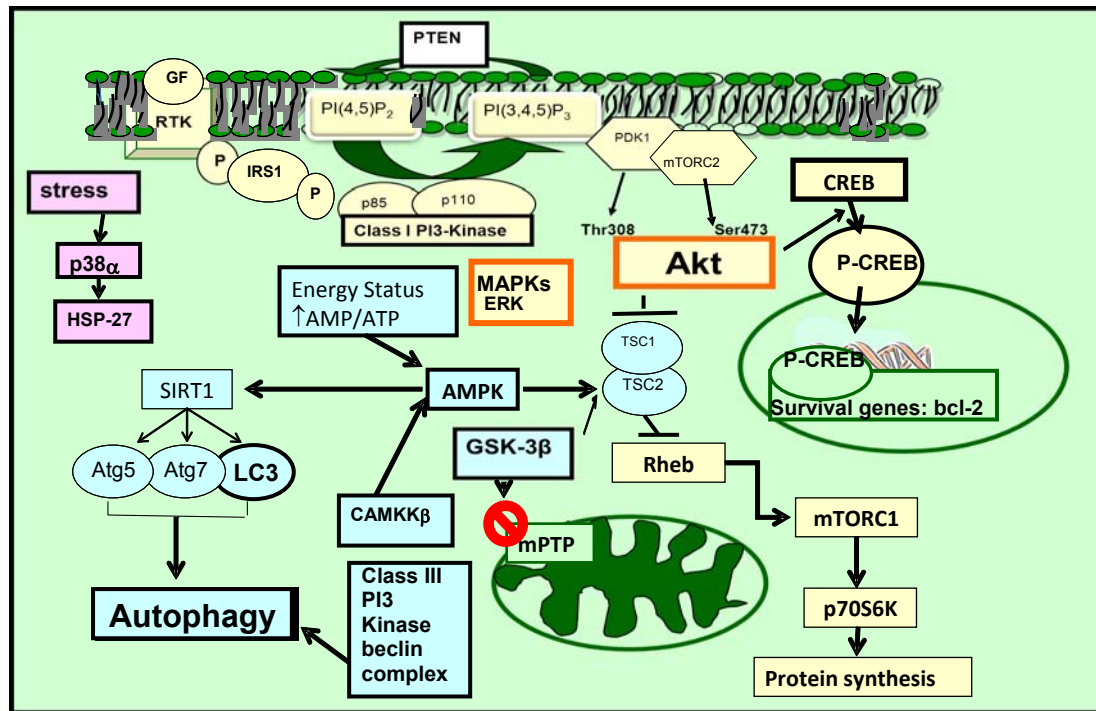
2010]. Autophagy during reperfusion is believed to be detrimental, unless it is also associated with AMPK.

In the present study, the activation of autophagy in IPC at 5 minutes reperfusion was accompanied by activation of AMPK, an inhibitor of the mTOR pathway, suggesting the induction of protective autophagy at this timepoint. Activation of autophagy in the Non-IPC hearts at 10 minutes reperfusion, may represent a compensatory mechanism to maintain homeostasis during the early stages of ischaemia/reperfusion damage. Attenuation of autophagy at 10 minutes reperfusion in IPC may indicate a switch between protective autophagy, observed at 5 minutes reperfusion to possibly impaired or detrimental autophagy observed at 30 minutes reperfusion in IPC. Autophagy in IPC at 30 min reperfusion was accompanied by activation of caspase-3. This may suggest impaired or detrimental autophagy at this timepoint. Various studies demonstrated that apoptosis and autophagy are not always mutually exclusive [Ryter et al., 2010]. These 2 processes can sometimes, as a result of impaired or excessive autophagy occur simultaneously in the same cell [Galluzzi et al., 2008; Maiuri et al., 2007; Kroemer & Levine 2008; Nishida et al., 2008]. Various studies indicated that excessive autophagy directly affects cell death pathways [Levine & Yuan 2005; Galuzzi et al., 2008; Maiuri et al., 2007] and can lead to cardiac diseases [Nishino et al., 2000; Tanaka et al., 2000]. Impaired autophagy was demonstrated in hypertrophied hearts [Dammrich & Pfeifer, 1983]. Chronic ischaemia resulted in excessive autophagy and the expression of autophagic markers in cardiac tissue, reached a maximal response after repeated cycles of ischaemia [Yan et al., 2005].

To conclude, we propose the following activation patterns (signaling pathway) of the kinases investigated in this part of the study in IPC during early (5 minute) reperfusion as indicated in Figure 3.5.

The PI3-K pathway is triggered by binding of growth factors (GFs) to the receptor tyrosine kinase (RTK), thereby activating PI3-K. Activated PI3-K converts PIP<sub>2</sub> to PIP<sub>3</sub> which recruits phosphoinositide-dependent kinase1 (PDK1) and PKB/Akt (part of the RISK pathway) to the cell membrane. PKB/Akt is then phosphorylated by PDK1 on Threonine<sup>308</sup> and by mTOR2 on Serine<sup>473</sup>. Activated PKB/Akt in

turn, phosphorylates and inactivates tuberous sclerosis complex (TSC) 1/2 leading to activation of Rheb and consequently mTORC1. AMPK, the energy sensor sensitive to changes in the intracellular ATP/AMP ratio, directly phosphorylates TSC2, thereby providing the priming phosphorylation for subsequent phosphorylation of TSC2 by glycogen synthase kinase 3 (GSK-3) to inhibit mTORC1 signalling. When AMPK phosphorylates and activates TSC1/2 complex, the mTOR1-mediated inhibition of autophagy is relieved and protective autophagy is induced. AMPK can also activate SIRT1 which can deacetylate and activate several autophagy related proteins such as Atg5, Atg7 and LC3. Mitogens and cellular stress activate the MAPKs which include ERK (part of the RISK pathway) and p38 MAPK. P38 MAPK activates MAPKAP2 which in turn can activate HSP-27 which protects the heart from ischaemia/reperfusion damage by stabilization of the actin cytoskeleton. In addition, HSP-27 decreases the intracellular ROS content and prevent apoptotic cell death, by binding directly to cytochrome c [Paul et al., 2002; Samali et al., 2001].



**Figure 3.5 Proposed activation patterns of the kinases during reperfusion after IPC compared to Non-IPC hearts.**

The PI3-K pathway is triggered by binding of growth factors (GFs) to the receptor tyrosine kinase (RTK), thereby activating PI3-K. Activated PI3-K converts  $PIP_2$  to  $PIP_3$ , which then recruits phosphoinositide-dependent kinase1 (PKD1) and PKB/Akt to the cell membrane. PKB/Akt (part of RISK pathway) is then phosphorylated by PDK1 on Thr<sup>308</sup> and by mTORC2 on Ser<sup>473</sup>. Activated PKB/Akt, in turn, phosphorylates and inactivates tuberous sclerosis complex (TSC) 1/2, leading to activation of Rheb and consequently of mTORC1. AMPK, a cellular energy sensor of changes in the intracellular ATP/AMP ratio, directly phosphorylates TSC2, thereby providing the priming phosphorylation for subsequent phosphorylation of TSC2 by glycogen synthase kinase 3 (GSK3) to inhibit mTORC1 signaling. When AMPK phosphorylates and activates TSC1/2 complex, the mTORC1-mediated inhibition of autophagy is relieved, and autophagy is induced. AMPK can also activate SIRT1 which can deacetylate and activate several autophagy-related proteins such as Atg5, Atg7 and LC3. Mitogens and cellular stress activate the MAPKs, which include ERK (RISK pathway) and p38 MAPK. p38 MAPK can activate HSP-27 which protects the heart from reperfusion-induced damage.



## Chapter 4

### Two-Dimensional Gel Electrophoresis (2-DE Proteomics)

#### 4.1) Introduction

In the past decade, there has been remarkable progress in the field of protein technologies. Mass spectrometry (MS) has emerged as the preferred analytical tool for in-depth studies of the components of biological systems [Cravatt et al., 2007]. Important insights have been gained by these studies regarding functions of proteins as well as regulation of molecular complexes and their biological pathways. Mass spectrometry based proteomics is now well recognized as a powerful technology that has the ability to advance our understanding of the dynamics and complex nature of the proteome [Parag & Kuster, 2010].

Proteomic studies are generally accomplished by combining two-dimensional gel electrophoresis (2-DE) with mass spectrometry. Although the basic 2-DE technique was developed 30 years ago, it is still acknowledged as the core technology of choice in the majority of proteomic projects [Jiang & Wang, 2012]. 2-DE is a gel-based, label-free proteomics technique which allows separation and visualization of proteins, followed by mass spectrometry for peptide analysis. Peptide files generated from the MS analysis are subjected to different database searches to identify the corresponding proteins [Gygi et al., 2000]. The major advantage of the 2-DE technique is that the gel analysis is broad-based and effectively unbiased from the researcher's point of view. Since the 2-DE approach is non-targeted, it can reveal novel discoveries [Lindsey et al., 2012]. It is an established technology and separates intact proteins. Another strength of this technique is that it provides apparent molecular weight and information regarding the iso-electric point (pI) of proteins, therefore allowing the detection of post translational modifications. In addition, detailed image analysis of gel spots using software such as PDQuest, allows for the identification of significant differences between comparison groups. This affords the benefit that subsequent mass spectrometry analysis only have to be performed on these spots [Lindsey et al., 2012]. The limitations of this approach is the dynamic range which is  $10^4$ . Several



strategies can be employed to overcome this: 1) the use of narrow-range pH strips to ensure full proteomic coverage; 2) optimizing sample solubility by choosing the correct sample detergent combination during tissue homogenization; 3) to enrich low abundance proteins by pre-fractionation (fractionation into different cell fractions i.e. cytosolic or membrane fractions and also fractionation into different organelle fractions i.e. mitochondrial fraction); 4) to include technical replicates (each sample has to be run in triplicate). Another disadvantage of 2D is that in the absence of any automation (PDQuest connected to a robotic spot cutter attached to LC-MS) it is a labour intensive technique.

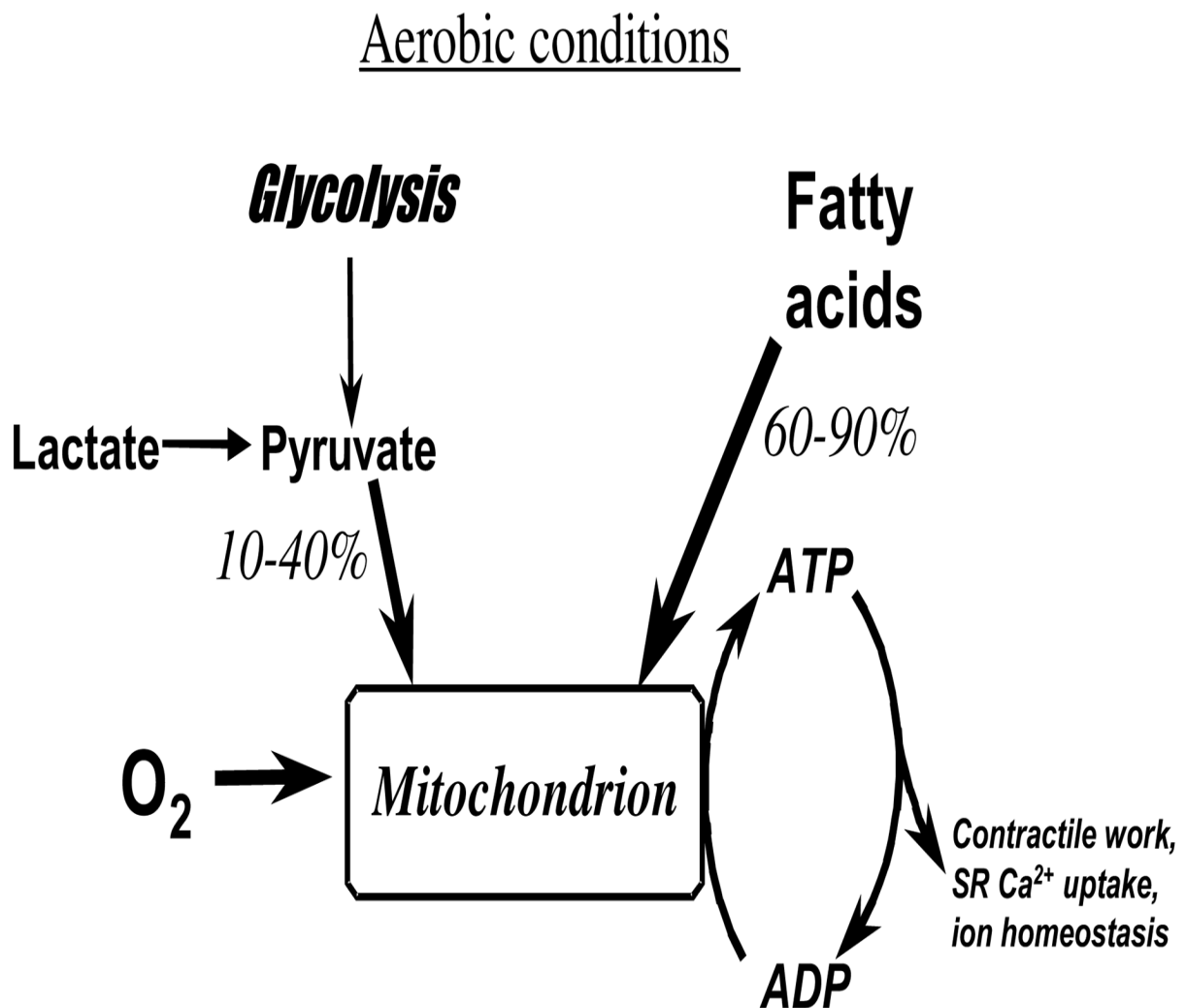
2D-DIGE (2D-Differential Gel Electrophoresis), also a gel-based proteomics technique, has been developed recently and implemented as another powerful tool to study cardiovascular diseases [Lilley & Friedman, 2004]. The advantages of 2D-DIGE is that up to three samples can be separated on one gel. It is therefore less labour intensive than 2D. The samples are fluorescently labeled with three different fluorochromes; Cy2, Cy3 and Cy5. All 3 samples are mixed and run on the same gel with an internal standard. 2D-DIGE allows the reliable quantification of difference in protein expression as low as 10%. The disadvantage of 2D-DIGE is that membrane proteins are poorly represented by this technique and low-abundance proteins are difficult to identify [Lilley & Friedman, 2004; Mayr M et al., 2011].

As part of this research project a 2D proteomics study was conducted on the mitochondrial and cytosolic fractions of rat heart tissue. Before discussing the results of the present study, an overview of the myocardial metabolism will be given.

## 4.2) Energy metabolism in the aerobic heart

The high energy demand of the heart is met by utilizing a variety of carbon substrates, including free fatty acids (FFAs), carbohydrates, amino acids and ketone bodies (Figure 4.1) [Stanley et al., 2001]. Free fatty acids and carbohydrates are the major substrates from which the heart derives most of its energy. Under normal, aerobic conditions, 50-70% of the total energy is obtained from fatty acids, while the majority of the rest is obtained from carbohydrates (mainly glucose and lactate).

Approximately 60-70% of the ATP obtained is used to supply the energy required for contractile shortening, while the rest (30-40%) is mainly used for the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase and other ion pumps [Suga et al., 1990; Gibbs, 1978]. Mitochondrial oxidative phosphorylation is fuelled with energy generated from electrons, transferred from carbon fuels by dehydrogenation reactions. The reducing equivalents NADH and  $\text{FADH}_2$  are subsequently produced mainly by the beta-oxidation pathway and to a lesser extent from glycolysis (Figure 4.1) [Stanley et al., 2005]. A stoichiometric link exists between the rate of oxidation of carbon fuels, oxygen consumption, ATP hydrolysis, flux through the electron transport chain (ETC), oxidative phosphorylation, actin-myosin interaction and the external contractile power generated by the heart [Stanley et al., 2005]. Therefore, an increase in contractile power, results in a concomitant increase in all the components in the system. The regulation of myocardial metabolism is linked to arterial carbon substrate concentration, hormone concentrations, inotropic state and coronary flow [Taegtmeyer et al., 2002; Opie 1991].



**Figure 4.1 Cardiac energy metabolism under normal aerobic conditions.**

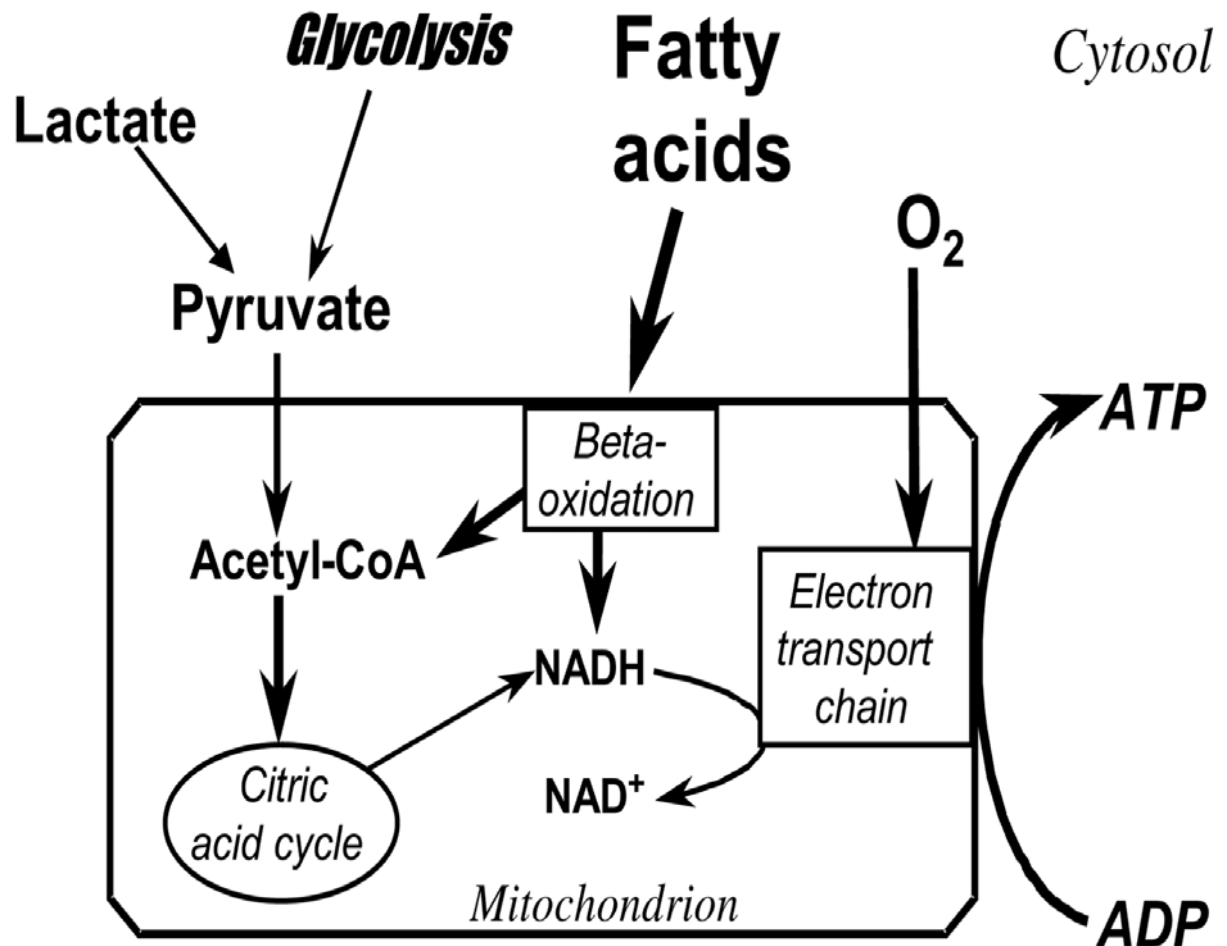
Fatty acids are the primary source of energy for the heart, supplying 60–90% of the energy for adenosine triphosphate (ATP) synthesis. The balance (10–40%) comes from the oxidation of pyruvate formed from glycolysis and lactate oxidation. Almost all of the ATP formation comes from oxidative phosphorylation in the mitochondria; only a trivial amount of ATP (<2% of the total) is synthesized by glycolysis. ADP=adenosine [Stanley, 2001]

### 4.2.1) Glycolysis

Glycolysis (“lysis of glucose”) is the biochemical process by which glucose is metabolized to pyruvate leading to the production of acetyl-CoA, which is further metabolized in the citric acid (Krebs) cycle. All the enzymes of the glycolysis pathway are located in the cytosol. Glycolysis is a unique pathway, since it can utilize oxygen when under aerobic conditions, but it can also function in the absence of oxygen (anaerobic conditions).

The function of the citric acid cycle (Krebs cycle) is to act as the final common pathway for the oxidation of carbohydrates, proteins and lipids (Figure 4.2). The enzymes of the citric acid cycle are located in the mitochondrial matrix. The Krebs cycle forms an integral part of a process in which much of the free energy liberated during oxidation of carbohydrates, lipids and amino acids is made available. During the course of oxidation of acetyl-CoA in the cycle, reducing equivalents in the form of hydrogen or electrons are formed as a result of the activity of specific dehydrogenases.

These reducing equivalents then enter the respiratory chain, where large amounts of ATP are generated in the process of oxidative phosphorylation. This process is aerobic and therefore absence or partial deficiency of oxygen causes total or partial inhibition of the cycle.



**Figure 4.2 Mitochondrial energy metabolism.**

Reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (not shown) transfer electrons from fatty acids, glucose and lactate to the electron transport chain. The process of oxidative phosphorylation is driven by the electron transport chain, which takes the energy from the oxidation of fatty acids, glucose and lactate, primarily via NADH. ADP=adenosine diphosphate; ATP=adenosine triphosphate; CoA=coenzyme A; NAD<sup>+</sup> = nicotinamide adenine dinucleotide [Stanley, 2001].

#### 4.2.2) $\beta$ -oxidation in the aerobic heart

Within the circulation, fatty acids are tightly bound to albumin and represent an important, readily available energy source for certain organs like the heart. Entry of fatty acids into the myocardial cell is presently thought to be mediated by several proteins including fatty acid binding proteins (FABPs) and a myocardial-specific integral membrane transporter (fatty acid translocase or FAT). The non-enzymatic FABP also serves as a facilitator of intracellular transport of relatively insoluble long-chain fatty acids to sites of metabolic utilization (e.g. mitochondria). In mammals, the FABP content in skeletal and cardiac muscle is related to the fatty acid oxidation capacity of the tissue [Glatz & Storch, 2001].

Once in the cytoplasm, long-chain fatty acids (LCFAs) may be transported by heart-type fatty acid binding protein (H-FABP) [Binas et al., 1999] and are rapidly esterified to acyl-CoA by long-chain acyl-CoA synthase (LCAS). Transport of LCFAs into the mitochondria occurs via transesterification by carnitine palmitoyltransferase I (CPT-I) and subsequent translocation across the inner mitochondrial membrane facilitated by carnitine/acyl-carnitine translocase. CPT-I catalyzes a key rate-limiting step in mitochondrial fatty acid flux and, as such, is highly regulated at gene transcriptional level [Brown et al., 1995; Brandt et al., 1998; Mascoró et al., 1998; Steffen et al., 1999] as well as through post-translational control via reversible binding of the inhibitor, malonyl-CoA, the first committed intermediate in the pathway of fatty acid synthesis [McGarry et al., 1989]. Upon translocation across the inner mitochondrial membrane, long-chain acylcarnitines are re-esterified to acyl-CoA derivatives by CPT-II and enter the  $\beta$ -oxidation spiral. The initial step in the oxidative spiral is catalysed by: (1) a family of acyl-CoA dehydrogenases (AD) specific for very long-chain (VLCAD), long-chain (LCAD), medium-chain (MCAD) and short-chain (SCAD) acyl-CoA substrates. Subsequent steps involve: (2) hydration by enoyl-CoA hydratase; (3) a second oxidation by a 3-hydroxyacyl-CoA dehydrogenase; and then (4) a thiolytic cleavage by 3-ketoacyl-CoA thiolase to yield acetyl-CoA and shortened acyl-CoAs destined for additional rounds of oxidation. Steps 1 and 3 of the fatty acid oxidation (FAO) cycle generate electrons which are ultimately transferred to

the electron transport chain where ATP is produced in the presence of oxygen (i.e. oxidative phosphorylation). The acetyl-CoA end-product is oxidized via the tricarboxylic acid (TCA) cycle.

The process of fatty acid oxidation is termed  $\beta$ -oxidation, since it occurs through the sequential removal of 2-carbon units by oxidation of the  $\beta$ -carbon position of the fatty acyl-CoA molecule. Each round of  $\beta$ -oxidation produces NADH, FADH<sub>2</sub> and acetyl-CoA. The acetyl-CoA, the end product of each round of  $\beta$ -oxidation, enters the TCA cycle where it is further oxidized to CO<sub>2</sub> with the concomitant generation of NADH, FADH<sub>2</sub> and ATP. The NADH and FADH<sub>2</sub> generated during fatty acid oxidation and acetyl-CoA oxidation in the TCA cycle will subsequently enter the respiratory pathway for the production of ATP. Consequently, the oxidation of fatty acids yields more energy per carbon atom than does the oxidation of carbohydrates. However, while fatty acids produce more ATP during complete aerobic oxidation than glucose, this occurs at the expense of a higher rate of oxygen consumption. The supply of oxygen is an important determinant of myocardial fuel utilization.

#### **4.2.3) Energy metabolism in the ischaemic and reperfused heart**

All of the ATP produced from fatty acid oxidation is dependent on the presence of oxygen. In contrast, ATP production from glucose originates from both glycolysis (which is not dependent on oxygen) and glucose oxidation (which is dependent on the presence of oxygen). During ischaemia, due to restricted oxygen supply to the muscle, both fatty acid and carbohydrate oxidation decreases and ATP production is impaired (Figures 4.3; 4.4) [Lopaschuk, 1997].

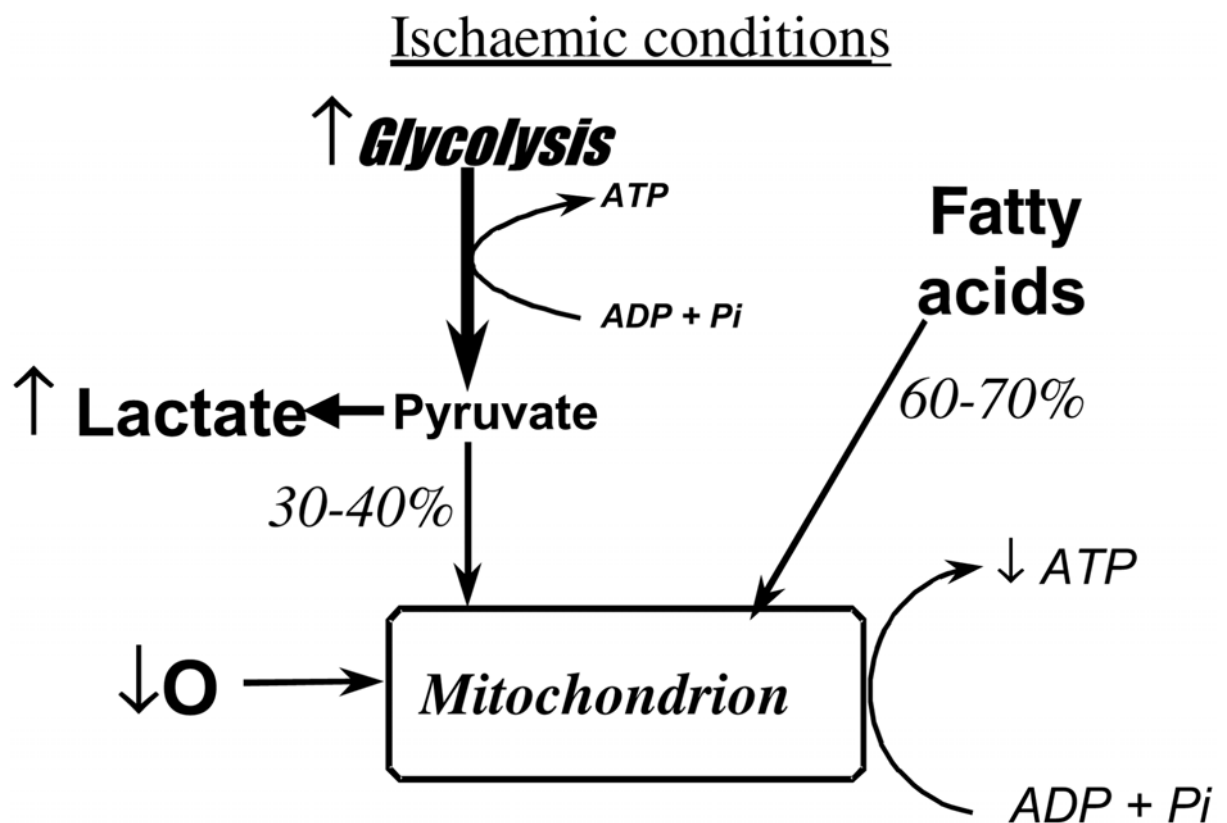
Glycolysis, a minor source of ATP in the aerobic heart, becomes a more significant source of energy during ischaemia [Olivier & Opie, 1994]. However, pyruvate produced from glycolysis is converted to lactate rather than being completely metabolized to CO<sub>2</sub> and H<sub>2</sub>O in the mitochondria. This not only results in the accumulation of lactate but also H<sup>+</sup>s, since H<sup>+</sup> produced from hydrolysis of glycolytically derived ATP are not taken up by the mitochondria (with pyruvate)

where it eventually is used for H<sub>2</sub>O production. This results in a reduction in intracellular pH, the magnitude of which depends on the severity of ischaemia [Kloner & Jennings, 2001; Liu et al., 2001].

During reperfusion of previously ischaemic muscle, there is the possibility of an impaired post-ischaemic functional recovery due to a delayed recovery of metabolism [Schwaiger et al., 1985]. However, in the reversibly injured myocardium, fatty acid oxidation rapidly recovers during reperfusion and becomes the dominant source of energy replenishing the ATP pool in the heart [Belke & Lopaschuk, 1997]. Glucose oxidation, on the other hand remains suppressed due to the fact that increased fatty acid oxidation inhibits glucose oxidation via the well known Randle cycle (increase in fatty acid oxidation inhibits glucose oxidation in the muscle via inhibiting the rate limiting pyruvate dehydrogenase complex of the glucose oxidation pathway) [Kantor et al., 2001]. Interestingly, glycolysis is unaffected by these high rates of fatty acid oxidation and remains elevated during reperfusion resulting in an increase in the uncoupling of glycolysis from glucose and a continued H<sup>+</sup> accumulation [Liu et al., 1996]. Activation of AMPK during and following ischaemia is an important contributor to these high rates of both glycolysis and fatty acid oxidation.

Proton accumulation during ischaemia and reperfusion can alter cardiac efficiency by a number of potential mechanisms. It can lead to intracellular Na<sup>+</sup> accumulation via Na<sup>+</sup>/H<sup>+</sup> exchange and a subsequent intracellular Ca<sup>2+</sup> accumulation via Na<sup>+</sup>/Ca<sup>2+</sup> exchange [Liu et al., 1996]. Sudden rises in intracellular Ca<sup>2+</sup> could potentially cause cell death [Liu et al., 1996]. Proton accumulation can also result in a decreased efficiency of the contractile proteins, impair functional recovery during reperfusion and decrease cardiac efficiency (cardiac work/myocardial O<sub>2</sub> consumption). Liu and co-workers [1996] have also demonstrated that decreasing H<sup>+</sup> production (by improving uncoupling) and/or inhibiting Na<sup>+</sup>/H<sup>+</sup> exchange, improves functional recovery as well as cardiac efficiency during reperfusion. Thus, altered metabolism during ischaemia is an important contributor to post-ischaemic functional impairment of the heart.

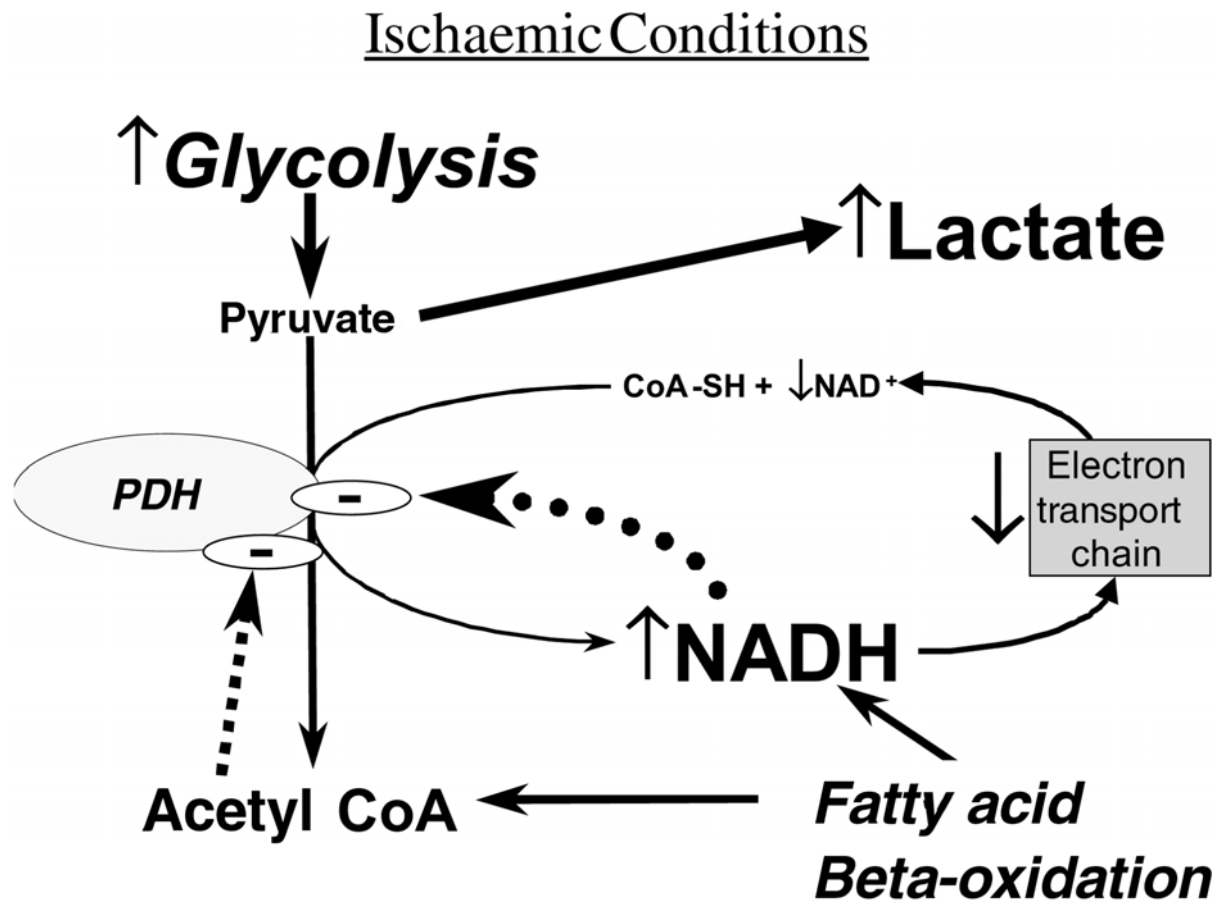




**Figure 4.3**

**Cardiac energy metabolism during ischaemia of moderate severity (approximately 40% of normal blood flow).**

The up and down arrows indicate the changes compared with normal aerobic conditions. Relative to aerobic conditions, ischaemia results in an increase in glycolysis without an increase in the rate of pyruvate oxidation, thus causing lactate to accumulate in the cell. Despite accelerated glycolysis and lactate production, the relatively high rate of residual oxygen consumption is fueled primarily by the oxidation of fatty acids. ADP=adenosine diphosphate; ATP=adenosine triphosphate [Stanley, 2001]



**Figure 4.4**

**Pyruvate oxidation during ischaemia.**

There is accelerated glycolysis and lactate production in the cytosol. In the mitochondria there is a rise in the ratio of reduced nicotinamide adenine dinucleotide (NADH) to oxidized nicotinamide adenine dinucleotide (NAD<sup>+</sup>) due to a decrease in oxygen consumption and continued fatty acid oxidation [Stanley, 2001].

#### **4.2.4) AMPK control of fatty acid metabolism during ischaemia/reperfusion**

AMPK, described as a “cellular fuel gauge”, shuts down the energy consuming processes and facilitates energy producing processes during various metabolic stresses [Hardie & Hawley, 2001]. Ischaemic stress in the heart leads to an increase in AMPK activity [Kudo et al., 1995, 1996]. AMPK modifies two important metabolic pathways during and post ischaemia, namely glycolysis and fatty acid oxidation. AMPK also inhibits creatine kinase and discourages the reverse reaction in which Cr is converted to PCr, and thus makes more ATP available for other critical functions of the cell [Ponticos et al., 1998].

AMPK belongs to a family of serine/threonine kinases which have highly conserved kinase domains [Lefebvre et al., 2001]. It was originally identified as a HMG CoA reductase kinase and an acetyl CoA carboxylase kinase [Carlson et al., 1973; Beg et al., 1978; Keith et al., 1979]. However, when it became clear that this kinase phosphorylated a number of proteins and was activated by 5'AMP, it was renamed as AMPK [Ferrer et al., 1985; Carling et al., 1987].

Under normal conditions, fatty acids are the preferred energy source of the myocardium contributing to 60-80% of the total ATP production [Belke & Lopaschuk, 1997]. Although fatty acid oxidation decreases during ischaemia, it rapidly recovers during reperfusion contributing to between 90-100% of the ATP production in the heart [Belke & Lopaschuk, 1997]. This increased use of fatty acids to support ATP production is due to an increase in circulating fatty acid levels, and to subcellular alterations in the control of fatty acid oxidation, where AMPK appears to play an important role.

In clinical conditions like myocardial infarction, or during and after cardiac surgery, serum fatty acid concentrations increase [Opie, 1975; Mueller & Ayres, 1987; Lopaschuk et al., 1994; Fragaso et al., 2002]. As a result, cardiac fatty acid supply and oxidation could increase several fold under these conditions. Inhibition of fatty acid oxidation protects the heart from detrimental effects of

heparin [Fragaso et al., 2002], implicating the detrimental effects of elevated fatty acid oxidation. Experimental evidence further supports that over-reliance on fatty acid oxidation is detrimental to the functional recovery of the heart during reperfusion following severe ischaemia [Lopaschuk et al., 1988; 1992]. Thus, it is important to understand the mechanisms responsible for alterations in fatty acid metabolism during ischaemia/reperfusion.

An important control site of fatty acid oxidation in the heart is at the mitochondrial membrane where fatty acids are transported across the membrane as acyl carnitine esters. This process is facilitated by acyl carnitine translocase and two carnitine palmitoyl transferases (CPT-I and CPT-II) (as discussed earlier). Once inside the mitochondria, acyl-CoA undergoes  $\beta$ -oxidation in the matrix producing several acetyl-CoA molecules which then enter the TCA cycle for complete oxidation. Transport of fatty acids by CPT-I across the mitochondria is the rate-limiting step in fatty acid oxidation [Kantor et al., 2001]. CPT-I, located on the outer mitochondrial membrane, is under the inhibitory control of malonyl-CoA [Kantor *et al.*, 2001]. Cellular malonyl CoA levels are in turn regulated by the rate of synthesis by acetyl-CoA carboxylase (ACC) and the rate of degradation by malonyl-CoA decarboxylase [Sakamoto et al., 2000]. AMPK has a potentially important role here, since it has been shown to regulate both ACC and MCD activities.

Thus, AMPK could be a potential target for metabolic modulation during ischaemia and reperfusion. It is also proposed that activating AMPK in the heart may be beneficial since it reduces lipid accumulation and therefore the lipotoxicity [Minokoshi et al., 2002]. However, it is important to note that activation of AMPK in the heart may also increase fatty acid oxidation and glycolysis which may have deleterious effects on the heart especially under conditions where plasma fatty acids are already elevated and where the heart utilizes exclusively fatty acids as its energy substrate (e.g. in diabetes and myocardial ischaemia).

### 4.3) Motivation leading to the present study

Although the protection elicited by IPC is a powerful response, our results demonstrated that some preconditioned hearts have a poor recovery after reperfusion. Since kinase activation (as accessed by Western blotting), does not always correlate with functional recovery during reperfusion (Chapter 5), it does not necessarily contribute to identify the underlying mechanisms involved or associated with poor recovery after IPC.

In view of the above, our aims were to identify the underlying mechanisms involved contributing to poor recovery of the IPC hearts during reperfusion by implementing a more sensitive technique and allowing us to study more than one protein at a time. In order to achieve this, we utilized the 2D proteomics technique. This technique is well established in our lab and implementing the latest IPG strip technology and precast gradient gels during protein separation, combined with ESI-MS has proven to yield satisfactory results when separating mitochondrial proteins.

Emerging studies suggest that events occurring during the early post-ischaemic myocardial reperfusion phase may underlie the cardioprotection elicited by IPC [Hauenloy et al., 2005]. Therefore, we decided to study the 15 min reperfusion time point. The patterns of kinase activation of the 15 min reperfusion time point are shown in chapter 3. Another benefit of studying this time point is that functional recovery can be recorded after 15 min reperfusion, thus enabling us to correlate functional recovery with biochemical processes in the cell, whereas at 5 and 10 min reperfusion the heart is still perfused in retrograde mode.

Myocardial cell death due to ischaemia/reperfusion injury is a major cause of morbidity and mortality nationwide [Murphy & Steenbergen, 2008]. Mitochondria are considered the most important organelle of eukaryotic cells and are the major contributors in many cellular as well as extracellular regulatory functions that affect the differentiation and survival of living organisms [Jiang & Wang, 2012]. The primary function of mitochondria is to converge organic materials into cellular energy in the form of ATP. Mitochondria play an important role in many crucial metabolic tasks, such as regulation of the cellular redox state, heme and steroid

synthesis, urea cycle, fatty acid oxidation and heat production [Kim et al., 2006]. When oxygen availability becomes limited, as during long periods of ischaemia alterations to mitochondrial structure can result in mitochondrial dysfunction. Mitochondrial structural alterations and dysfunction has proved to have deleterious effects on cellular level and have been implicated in numerous forms of diseases, including heart disease, ischaemia/reperfusion injury and neurological diseases [Hirano et al., 2001; Beal et al., 1997; Yarian 2005]. Ischaemic heart disease (IHD) represents a group of pathological disorders, characterized by insufficient perfusion (oxygen supply) of the heart [Cadenas et al., 2010]. The production of ROS by the mitochondria is an important feature of ischaemia. During ischaemia ROS are generated in the myocardium and the major sites of ROS production in the mitochondria are complexes I and III of the ETC [Zweier & Talukder, 2006; Solani & Harris, 2005; Pacher et al., 2006]. Upon reperfusion, the production of ROS initiated by ischaemia is exacerbated. This overproduction of ROS coupled with an increase in intracellular calcium is thought to be the main cause of reperfusion injury [Cardenas et al., 2010]. The ischaemic heart is therefore an important model for studies of cardiovascular disease [Jiang & Wang, 2012]. The rapid emerging field of proteomics can now provide new insight into the identification and characterization of mitochondrial proteins in these processes. In the past mitochondria have been studied using classical biochemical methods, but these studies focused only on one particular protein involved in the disease processes. Proteomic techniques allow more in-depth studies involving the whole mitochondrial proteome. A search of the literature revealed a lack of proteomics studies involving preconditioning in the isolated working heart during reperfusion. Proteomic based studies in the heart however, have been utilised to investigate the following aspects; oxidative stress in H9C2 cardiomyocytes [Chou et al., 2010]; myocardial ischaemia [Peronnet et al., 2006; Kim et al., 2006; Koomen et al., 2006], dilated cardiomyopathy [Gramolini et al., 2008; Weekes et al., 1999; Isserlin et al., 2010], hypertrophic cardiomyopathy [Buscemi et al., 2005] and heart failure [Chen et al., 2008; Wei et al., 2009; Cieniewski-Bernard et al., 2009; Weger et al., 2011; Dai et al., 2012; Zhang et al., 2011]. The results of some of these studies will be summarized in the Discussion sections (4.7 and 4.8).

In view of the above our aims were, to identify the underlying mechanisms involved contributing to poor recovery of the IPC hearts during reperfusion by:

(1) comparing the protein expression profiles of the mitochondrial fractions of rat heart tissue between IPC and Non-IPC hearts; (2) comparing the protein expression profiles of the cytosolic fractions of rat heart tissue between IPC and Non-IPC, by utilizing the 2D proteomics technique (Materials and Methods, Chapter 2).

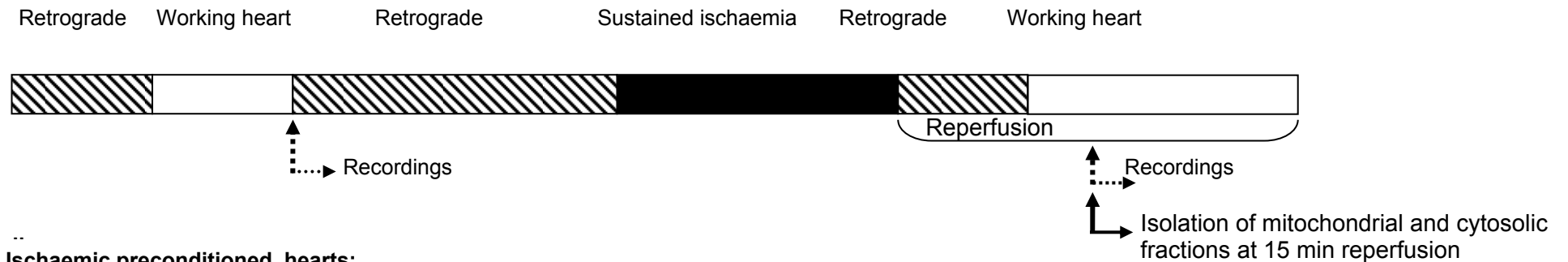
#### **4.4) Methods**

For the proteomic study, the following experimental protocol was used:

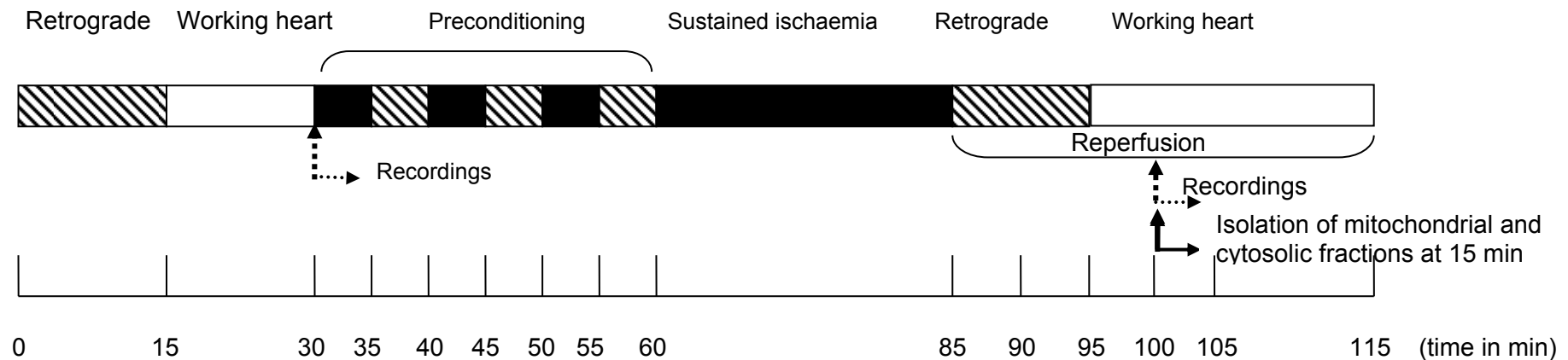
(i) Non-preconditioned hearts (Non-IPC). Isolated rat hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde). These hearts were subsequently subjected to 25 min sustained global ischaemia followed by 15 min reperfusion (10 min retrograde perfusion, followed by 5 min working heart) (Figure 4.5). After 15 min reperfusion hearts were snap-frozen and mitochondrial and cytosolic fractions were isolated using two different kits as described in the methods section.



(ii) Ischaemic preconditioned hearts (IPC). Isolated rat hearts were stabilized for 30 min (15 min retrograde, 15 min working heart) and then preconditioned for 3x5 min global ischaemia, alternated with 5 min reperfusion. These hearts were subjected to 25 min sustained global ischaemia, followed by 15 min reperfusion (10 min retrograde perfusion, followed by 5 min working heart) (Figure 4.5). After 15 min reperfusion, hearts were snap-frozen and mitochondrial and cytosolic fractions were isolated using two different kits as described in the methods section.

**Non-preconditioned hearts:**



**Ischaemic preconditioned hearts:**

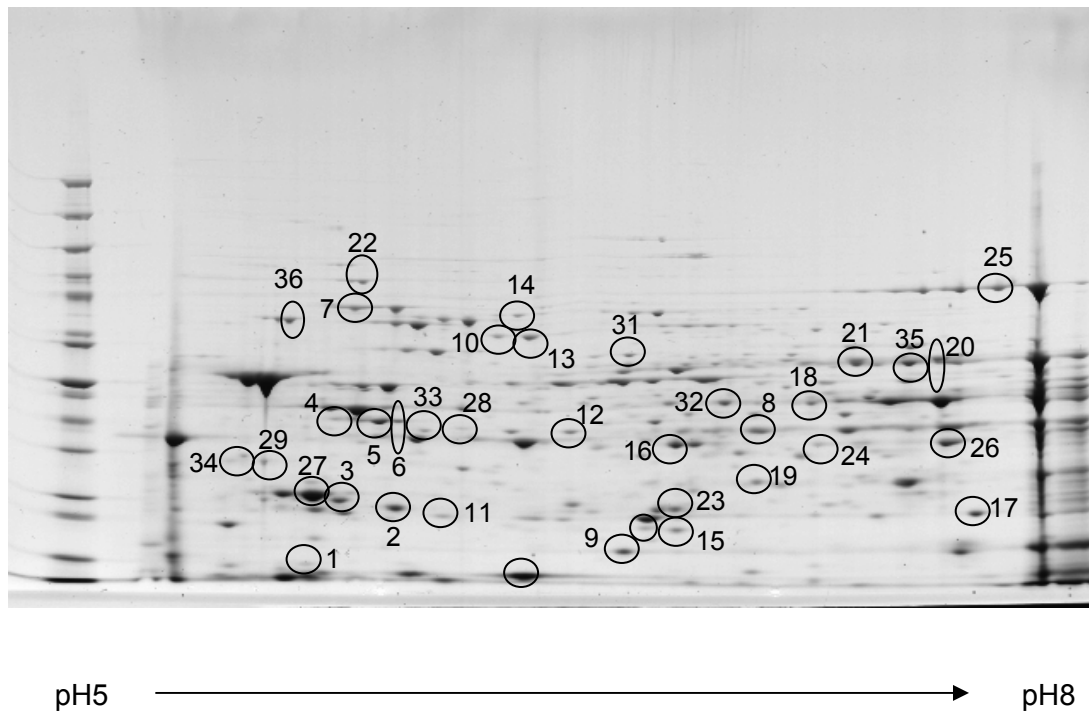


**Figure 4.5** Experimental protocol according to a timeframe. Mitochondrial and cytosolic fractions were isolated from heart tissue for the 2D proteomic study after 15 min reperfusion (indicated by the solid  arrows). Functional recordings were made indicated by the dashed arrows, ().



#### 4.5) Results: Cytosolic proteomics

Two dimensional proteomics of the cytosolic fraction of rat heart tissue highlighted several differences between Non-IPC and IPC rat hearts after 15 min reperfusion (Figure 4.6).



**Figure 4.6 A 2D gel-image of cytosolic proteins expressed in rat heart tissue.**

Differentially expressed protein spots ( $p < 0.05$ ) between Non-IPC and IPC were identified by PDQuest software and are indicated by numbers 1-18. These spots (spots of interest) were manually excised and subjected to in-gel digestion followed by Ms analysis. Peptide datasets were subjected to the Mascot search engine ([www.matrixscience.com](http://www.matrixscience.com)) and the corresponding proteins were identified, using the Swiss-Prot database.

The majority of proteins identified (61%) are involved in energy metabolism, 8% are structural proteins, 8% are heat shock proteins, while the rest have different functions. Several cytosolic proteins involved in energy metabolism were differentially expressed (statistically significant) between IPC and Non-IPC hearts after 15 min reperfusion (Table 4.1). These data show down regulation of two pivotal regulators of glycolysis, glyceraldehyde 3-phosphate (Table 4.1, spot 18) and pyruvate dehydrogenase (Table 4.1, spot 5) in the IPC hearts. Another energy regulator of glycolysis, pyruvate kinase, was up regulated in the IPC hearts (Table 4.1, spot 35).

The indirect energy-regulator lactate dehydrogenase (LDH), is downregulated (Table 4.1, spot 28) in the IPC hearts compared to the Non-IPC hearts, whereas some of the key regulators of electron transport in the electron transport chain (ETC) were down regulated in the IPC hearts.

Enzymes responsible for fatty acid  $\beta$ -oxidation (long chain specific acyl CoA dehydrogenase) Table 4.1, spot 21, in the mitochondria were up regulated in the IPC hearts, compared to the Non-IPC hearts. Interestingly, fatty acid binding protein (Table 4.1, spot 9), which is responsible for the transportation of fatty acids in the cell and the uptake of fatty acids into the mitochondria, was down regulated in the IPC hearts.

The expression of the Krebs cycle enzyme, isocitrate dehydrogenase [NAD] subunit (Table 4.1, spot 12), was decreased in the IPC hearts, while the expression of aconitase, another Krebs cycle enzyme (Table 4.1, spot 25), was up regulated in the IPC hearts compared to the Non-IPC hearts.

Heat shock protein-60 (HSP-60), Table 4.1, spot 8, was down regulated in the IPC hearts, while heat shock protein-beta-6 (Table 4.1, spot 15), was up regulated in the IPC hearts, compared to the Non-IPC hearts.

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
1	1004	278	Cytochrome C Oxidase	P11240	27%	6	- 2.86	Complex IV of the ETC
2	2102	395	Myosin light chain 3	P16409	72%	17	- 1.59	Cardiac muscle contraction
3	2201	400	NADH dehydrogenase (Ubiquinone)	P19234	47%	20	- 2.04	Complex 1 of ETC
4	2402	266	Prostaglandin reductase	Q5BK81	22%	9	- 1.82	Activates PPAR- $\gamma$
5	3401	472	Pyruvate dehydrogenase	O88989	39%	16	- 4	Glycolysis
6	3403	126	Troponin T, cardiac muscle	P50753	25%	12	- 9.1	Cardiac Muscle contraction

**Table 4.1** Proteins identified in the cytosolic fraction of rat heart tissue.

Data represent differentially expressed proteins between control (Non-IPC) and IPC ( $p < 0.05$ ) as identified by the Swiss-prot database. Only proteins that were significantly regulated are reported ( $p < 0.05$ ). Results are reported as fold increase or decrease compared to control (Non-IPC). SSP numbers are assigned by PDQuest Software (Bio-Rad, Hercules CA).

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
7	2906	396	Cytochrome b-c1 complex	Q68FYO	32%	20	- 1.61	Complex III of ETC
8	7301	573	HSP-60 Mitochondrial	P63039	43%	26	- 1.56	Mitochondrial protein import
9	4002	775	Fatty acid binding protein heart	P07483	66%	30	- 1.3	Transport of long chain fatty acids
10	4704	402	Prohibitin	P67779	52%	16	- 1.64	Inhibits DNA synthesis
11	3101	186	Phosphatidylethanolamine Binding protein	P31044	25%	3	1.6	Negative regulation of Raf/MAPK signaling
12	4305	258	Isocitrate dehydrogenase [NAD] subunit	Q99NA5	29%	15	- 1.14	Krebs cycle

Table 4.1 continued

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
13	4705	381	Acetyltransferase component of Pyruvate dehydrogenase Complex	P08461	19%	16	- 1.64	Glycolysis
14	4706	252	Myosin-6	P02563	12%	23	- 1.49	Muscle contraction
15	6103	107	HSP-beta 6	P97541	29%	6	1.89	Pre-slaughter stress
16	6302	388	Malate dehydrogenase	O88989	44%	21	1.38	Malate to oxaloacetate
17	9104	462	Alpha crystalline B-chain	Q9D6J6	59%	26	-1.72	Stabilizes actin cytoskeleton

Table 4.1 continued

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
18	7301	488	Glyceraldehyde 3- phosphate dehydrogenase	P04797	42%	17	- 1.56	Glycolysis
19	7203	383	Enoyl-CoA hydratase mitochondrial Proteosome	P14604	33%	13	1.31	Lipid metabolism
20	9705	376	ATP synthase	P15999	28%	22	- 2.86	ATP production
21	8501	585	Long chain specific acyl CoA dehydrogenase	P15650	42%	20	1.95	Fatty acid beta oxidation
22	2907	496	Transitional endoplasmic reticulum ATPase	P46462	33%	29	- 1.61	Reassembly of Golgi stacks after mitosis

Table 4.1 continued

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
23	5103	924	78 kDa Glucose regulated protein	P06761	46%	36	- 2.38	Assembly of proteins inside endoplasmic reticulum
24	7205	370	Delta (3,5)-Delta(2,4)-Dienoyl-CoA isomerase, mitochondrial	Q62651	27%	15	- 1.2	Fatty acid metabolism
25	9908	800	Aconitate hydratase, mitochondrial	Q9ER34	43%	42	1.73	Krebs cycle
26	8203	1108	Electron transfer flavoprotein subunit alpha	P56757	52%	36	-1.96	Transfer of electrons to main ETC
27	1102	434	Peroxiredoxin-6	O35244	59%	25	-2.33	Protects against Ischaemia/reperfusion injury

Table 4.1 continued

Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
28	2305	274	L-lactate dehydrogenase	P42123	39%	17	- 1.5	Glycolysis
29	0205	238	Annexin A5	P14668	39%	15	- 1.22	Anti-coagulant protein
30	4601	476	Alpha enolase	P04764	38%	19	-1.82	Glycolysis
31	4703	454	Protein disulfide-isomerase A3	P11598	42%	24	1.3	Heart failure
32	6402	479	Isovaleryl CoA dehydrogenase	O35244	43%	25	-1.89	Amino acid degradation

Table 4.1 continued

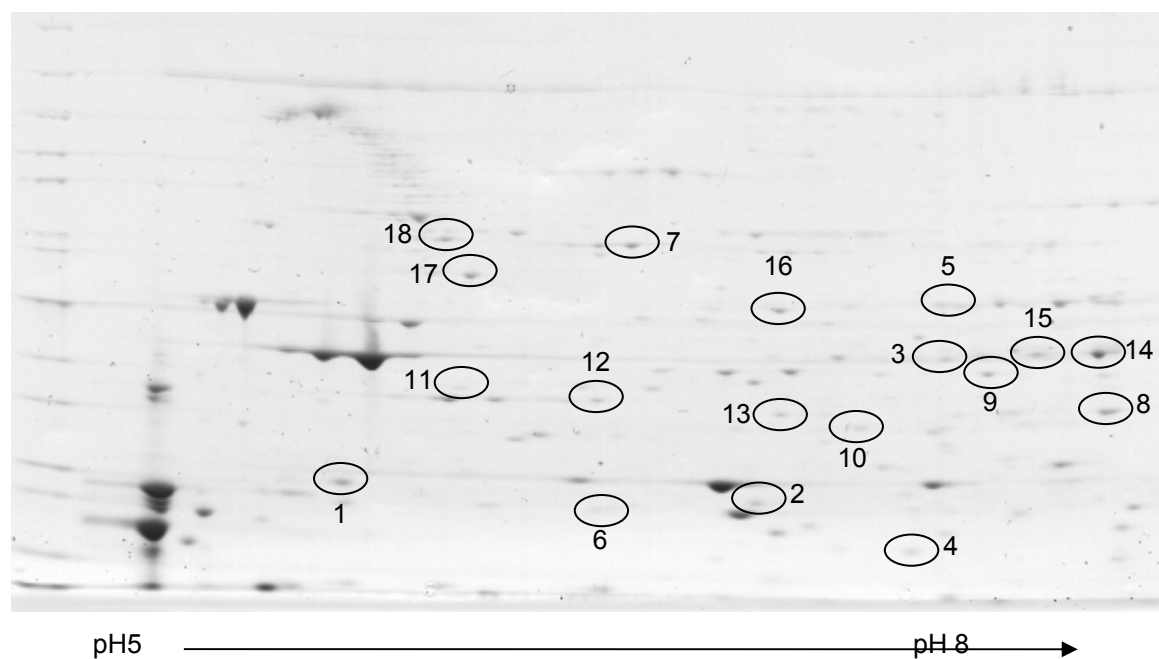


Spot	SSP	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
33	2304	130	Lactoylglutathione lyase	P42123	37%	11	- 1.5	Removes toxic aldehydes
34	0203	312	HSP -beta 1	P14668	39%	15	- 1.22	Anti-apoptotic
35	8704	576	Pyruvate kinase Isozymes M1/M2	P11980	46%	29	6.96	Glycolysis
36	1803	197	Protein DJ-1	O88767	33%	13	-2.38	Sensor of oxidative stress

Table 4.1 continued

#### 4.6) Results: Mitochondrial proteomics

2D proteomic analysis of mitochondrial proteins highlighted several differences between Non-IPC and IPC hearts after 15 min reperfusion (Figure 4.7).



**Figure 4.7** A 2D gel-image of mitochondrial proteins expressed in rat heart tissue. Differentially expressed protein spots ( $p < 0.05$ ) between Non-IPC and IPC were identified by PDQuest software and are indicated by numbers 1-18. These spot (spots of interest) were manually excised and subjected to in-gel digestion followed by Ms analysis. Peptide datasets were subjected to the Mascot search engine ([www.matrixscience.com](http://www.matrixscience.com)) and the corresponding proteins were identified, using the Swiss-Prot database.

Eighteen differentially expressed (statistically significant) proteins were identified between Non-IPC (control) and IPC hearts after 15 min reperfusion. These include 15 proteins involved in mitochondrial metabolism, three heat shock proteins, Annexin A3 and Mitofilin (Table 4.2). These data show both up- and down regulation of key regulators of electron transport in the ETC and mitochondrial ATP production.

Enzymes responsible for fatty acid  $\beta$ -oxidation (long chain specific acyl CoA dehydrogenase), in the mitochondria were up regulated in the IPC hearts, compared to the Non-IPC hearts. ATP synthase responsible for the generation of ATP from ADP was downregulated in the IPC hearts. Several enzymes responsible for the catabolism of amino acids were upregulated in the IPC hearts.

Spot	SSP	Mr (Da)	pI	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
1	0104	27703	6.23	194	Ubiquinone	P 12934	18	5	- 1.3	Complex I of ETC
2	5106	28563	7.14	40	Thioredoxin dependent peroxide reductase	Q920V6	4	2	1.13	Scavenger of mitochondrial generated hydrogen peroxide
3	7303	48242	7.63	248	Long chain specific Acyl-CoA dehydrogenase	P15650	13	5	1.6	Fatty acid beta oxidation
4	7102	20076	6.76	55	Alpha crystallin B chain	P23928	7	2	-1.2	Stabilization of actin cytoskeleton
5	7502	54574	7.69	40	Dihydrolipoyl dehydrogenase	P23434	2	2	1.41	Catabolism of glycine

**Table 4.2 Proteins identified in the mitochondrial fraction of rat heart tissue.**

Data represent differentially expressed proteins between control (Non-IPC) and IPC ( $p < 0.05$ ) as identified by the Swiss-prot database. Results are expressed as fold increase or decrease compared to control (Non-IPC). Only proteins that were significantly differently regulated are reported ( $p < 0.05$ ). SSP numbers are assigned by PDQuest.

Spot	SSP	Mr (Da)	pI	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
6	4204	18809	6.17	51	ATP Synthase D chain	P31399	21%	3	- 1.52	ATP synthesis
7	5607	74097	5.97	158	Heat shock protein 70	P63018	12%	7	- 1.82	Protein import
8	9306	35272	8.62	595	Electron transfer flavoprotein alpha	P13803	51%	16	2.23	Transfer electrons to main ETC
9	8306	45022	8.47	301	Short chain specific Acyl-CoA dehydrogenase	P15651	24%	8	1.95	Fatty acid metabolism
10	6208	33205	5.88	81	Mercaptopyruvate sulfurtransferase	P97523	9%	3	1.56	Catabolism of amino acids
11	4305	36874	5.7	222	L-lactate dehydrogenase	P42123	25%	9	1.65	Lactate to pyruvate

Table 4.2 continued

Spot	SSP	Mr (Da)	pI	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
12	3304	18809	6.17	51	Oxoisovalerate dehydrogenase	P21953	8	3	- 1.22	Conversion of alpha-keto acids to Acyl-CoA and CO <sub>2</sub>
13	4305	36569	5.96	68	Annexin A3	P14669	9	3	- 1.316	Cell regeneration
14	5301	40044	8.88	317	Isocitrate dehydrogenase [NAD]	P41562	33	19	1.31	Krebs cycle
15	9410	43246	6.58	394	Creatine kinase	P00564	32	13	1.82	Energy shuttle
16	9605	86121	7.87	118	Aconitase	Q9ER34	8	6	1.94	Krebs cycle

Table 4.2 continued

Spot	SSP	Mr (Da)	pI	Mascot score	Protein Name	Swiss Prot Accession Number	% Sequence Coverage	No of Peptides	Fold Increase or Decrease	Functional Group or Function
17	8402	17296	5.96	924	Nucleoside diphosphate kinase A	Q05982	2	13	1.5	Nucleotide biosynthesis
18	6502	57015	7.53	638	Aldehyde dehydrogenase	P11884	30	15	1.94	Omega oxidation of fatty acids
19	3602	61088	5.91	297	Heat shock protein 60	P 63039	25	15	-1.5	Folding of new proteins
20	3601	82247	618	173	Mitofilin	Q9NQR4	11	7	-1.724	Stabilization of actin cytoskeleton

Table 4.2 continued

## 4.7) Discussion: cytosolic proteomics

Some of the differentially regulated proteins (Table 4.1) will be individually discussed in this section.

### 4.7.1) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate (GAPDH) is a key energy regulator in the cardiac myocyte and the importance of this enzyme in regulating myocardial glycolysis has long been recognized. GAPDH catalyzes the oxidation of glyceraldehyde-3-phosphate (GAP) to 1,3-diphosphoglycerate by utilizing  $\text{NAD}^+$  as a redox factor and concomitantly reduces  $\text{NAD}^+$  to  $\text{NADH} + \text{H}^+$ . This makes GAPDH an energy regulator that is responsive to the energy demands of the cardiomyocyte through its sensitivity to the ratio of  $\text{NAD}^+$  to  $\text{NADH}$  (Mochizuki and Neely, 1979). The rate of glycolysis at high workloads is also regulated by GAPDH [Koayashi & Neely, 1979].

In the present study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was down regulated in the IPC hearts (Table 4.1, spot 18), suggesting that glycolysis is decreased in the IPC group. Down regulation of GAPDH may result in an accumulation of GAP, which contribute to enhanced triglyceride synthesis during reperfusion [Saddik & Lupaschuk, 1992]. It is well recognized that the production of fatty acids during reperfusion is detrimental to the post-ischaemic heart [Challoner & Steinberg, 1966]. The downregulation of GAPDH and subsequent attenuation of glycolysis is a direct effect of severe ischaemia [Rovetto et al., 1975]. Downregulation of GAPDH results in an increase in lactate with a subsequent decrease in the internal pH by  $\text{Na}^+/\text{H}^+$  and  $\text{Na}^+/\text{Ca}^{2+}$ . These processes lead to a rise in cytosolic calcium with adverse consequences which contribute to the overall mechanism of severe ischaemic damage [Cross et al., 1995]. Therefore the decreased expression of GAPDH in the IPC hearts can explain their poor recovery.

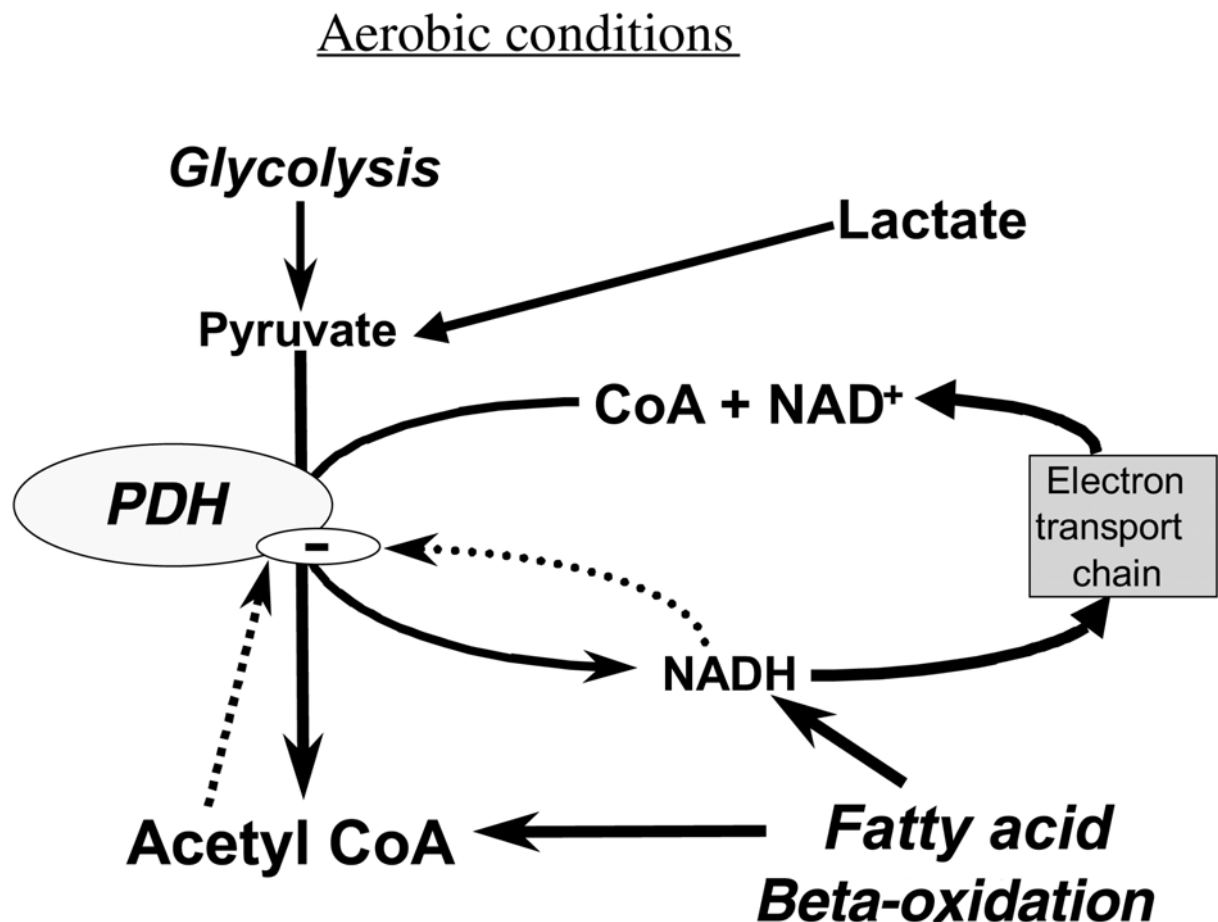


#### 4.7.2) Pyruvate dehydrogenase (PDH)

Pyruvate dehydrogenase forms part of a multi-enzyme complex collectively designated as the pyruvate dehydrogenase complex (PDC), located in the mitochondrial inner membrane. PDH stands at the crossroads of glycolysis in the cytosol and oxidation of carbohydrates in the mitochondria, thereby linking glycolysis and oxidative phosphorylation through the conversion of pyruvate to acetyl CoA (Figure 4.6) - this step commits pyruvate to oxidation in the Krebs cycle [Depre et al., 1999]. This increases the energy carrying capacity per glucose molecule almost fourteen-fold in the absence of the glycolytic NADH contribution to ATP. This conversion reaction is a complex process that involves the decarboxylation and oxidation of pyruvate to an acetyl molecule bound by lipoamide. The acetyl group is consequently transferred to CoA producing acetyl-CoA, followed by the formation of NADH [Stryer, 1998]. PDC is mainly inactive during fatty acid oxidation, but can be activated by enhanced glycolysis, as a result of increased work or in response to catecholamine stimulation [Depre et al., 1999]. It is important that this enzyme complex must be coordinated with the rate of glycolysis to avoid the buildup of pyruvate. This makes PDH a crucial energy modulator in the setting of glucose oxidation. When expression of this multi-enzyme complex is down regulated, as during ischaemia, lactate is formed especially when the flux of glycolysis is increased [Opie, 1998]. PDH is inhibited by its products acetyl CoA and NADH, therefore PDH and glycolysis are inhibited not only by a high energy potential but also under conditions of fatty acid oxidation.

In the present study, the expression of the PDH is down regulated (Table 4.1, spot 5 and spot 13) in the IPC hearts. As a result of this, the conversion of pyruvate to acetyl CoA will be retarded, since PDH is the link between glycolysis in the cytosol and oxidative phosphorylation in the Krebs cycle, as explained earlier. Pyruvate will therefore not be optimally oxidized to acetyl CoA in the Krebs cycle, which could be detrimental to the IPC hearts, as the Krebs cycle is fuelled by acetyl-CoA produced from pyruvate. In addition, the downregulation of GAPDH (section 4.7.1) impacts negatively on PDH as the accumulation of NADH<sub>2</sub> in the cytosol means more protons, thus intracellular acidosis is promoted. The result is further downregulation of PDH by NADH<sub>2</sub>, thereby

permitting entry into the Krebs cycle. Subsequently more lactate forms and the ratio of  $\text{NADH}_2$  to  $\text{NAD}$  increases further. This results in the accumulation of  $\text{NADH}_2$  in the mitochondria with adverse effects, characterized by downregulation of the Krebs cycle and an increase in mitochondrial calcium [Lehninger 1978]. These adverse processes can explain why the IPC hearts showed poor functional recovery.



**Figure 4.8 Regulation of pyruvate oxidation under normal aerobic conditions.** Pyruvate is formed in the cytosol from glycolysis and lactate oxidation and is converted to acetyl-coenzyme A (CoA) in the mitochondria by pyruvate dehydrogenase (PDH). The acetyl-CoA and reduced nicotinamide adenine dinucleotide (NADH) generated by fatty acid oxidation inhibit flux through PDH. Pharmacological inhibition of the rate of fatty acid oxidation removes inhibition of flux through PDH by NADH and acetyl-CoA and results in more pyruvate oxidation and thus more glucose and lactate uptake [Stanley, 2011].

### 4.7.3) Lactate dehydrogenase

Oxidation of lactate occurs through lactate dehydrogenase (LDH) to produce pyruvate. This is the reverse direction of the reaction whereby lactate is formed during hypoxia or ischemia. The resultant cytosolic NADH molecules have the same fate as that of the glycolytic NADH. The pyruvate is subsequently converted to acetyl-CoA by PDC with the formation of another NADH molecule. The net theoretical yield of ATP per lactate molecule oxidized is 14.75, half the amount produced by glucose oxidation [Opie, 1998].

Lactate is formed from pyruvate by LDH in the absence of oxygen, which is advantageous in sustaining glycolysis when oxygen levels become reduced. Thus, the formation of lactate facilitates a uninterrupted low ATP production during ischemia provided that the lactate is removed from the cytosol of the cardiomyocyte via the lactate-proton co-transporter (LPC) [Depre et al., 1999]. Indeed, during exercise or hypoxia, skeletal muscle and cardiac muscle become the major contributors to the elevation of blood lactate levels. In addition to the buffering effect on glycolysis, lactate can also be seen as a temporary mobile energy store to conserve energy. This is evident from the increased uptake of lactate from the blood by the myocardium when sufficient oxygen becomes available. Lactate is oxidized to pyruvate with a concomitant reduction of a NAD<sup>+</sup> molecule to NADH. The resultant pyruvate can then be oxidized in the mitochondria by the TCA cycle. Lactate competes remarkably well with glucose and fatty acids for oxidation by the heart if high levels of lactate are available [Opie, 1998; Lloyd et al., 2004; Liedtke et al., 1994].

In the present study, the expression of cytosolic LDH (Table 4.1, spot 28) was down regulated in the IPC hearts, compared to Non-IPC, suggesting that the conversion of lactate to pyruvate in the cytosol is retarded. This may result in an accumulation of lactate, intracellular acidosis and general disruption of cell homeostasis [Stanley et al., 2001]. The detrimental consequences of intracellular acidosis was explained in sections 4.7.1 and 4.7.2. These can account for failure of recovery of the IPC hearts.

#### **4.7.4) Pyruvate kinase (PK)**

This energy regulator plays an important role in the final step of glycolysis, where PK transfers one phosphate from phosphoenolpyruvate to ADP, thereby forming pyruvate and ATP. This irreversible reaction could play an important role in glycolytic regulation due to the sensitivity of PK to fructose 1,6 diphosphate (F1,6DP). Increased production of F1,6DP by increased activity of PFK1 enhances the activity of PK and thus pyruvate production (Kiffmeyer et al., 1991). This complimentary feed-forward reaction of energy regulators synchronizes the activity of PFK1 and PK, thereby preventing the accumulation of glycolytic intermediates during normoxia [Depre et al., 1999]. However, during ischemia, pyruvate accumulation could result in product-induced inhibition of PK that may retard the activity of PK and thus the rate of glycolysis. Removal of this glycolytic byproduct from the cytosol to the mitochondria is mediated by the PDH, which therefore has a regulatory role in glycolysis. In contrast, during ischemia, PDH becomes inhibited and the role of pyruvate removal is conferred to LDH [Kerbey et al., 1976; Opie, 1998].

In the present study the expression of PK (Table 4.1, spot 35) is increased in the IPC hearts, with the resulting formation of pyruvate in the cytosol. Since expression of PDH is down regulated in the IPC hearts the removal of pyruvate from the cytosol to the mitochondria (by PDH) will be retarded. Since the expression of LDH is also down regulated in the IPC hearts, pyruvate removal by LDH from the cytosol to the mitochondria will also be inhibited. As a result of this, pyruvate will accumulate in the cytosol.

#### **4.7.5) Complex I, III and IV of the Electron transport chain**

Mitochondria contain a series of catalysts known as the electron transport chain (ETC) that collects and transports reducing equivalents in a final common pathway to direct them in a final reaction with oxygen leading to the formation of water. These catalysts comprises of NAD-linked dehydrogenase systems, flavoproteins and cytochromes. Also present in the ETC is the machinery responsible for trapping the generated free energy as high energy phosphate.

Ubiquinone or Q (also known as Complex I or coenzyme Q) is an additional carrier present in the ETC linking the flavoproteins to cytochrome b. Q exists in mitochondria in the oxidized quinone form under aerobic conditions and in the reduced quinol form under anaerobic conditions. Q is a constituent of mitochondrial lipids and the more mobile component of the ETC enabling it to collect reducing equivalents from the more fixed flavoprotein complexes and pass them on to the cytochromes. In the present study, expression of four electron carrier proteins of the ETC are down regulated in the IPC hearts; Complex I or Q (Table 4.1, spot 3), Complex III (Table 4.1, spot 7), Complex IV (Table 4.1, spot 1) and electron transfer flavoprotein (Table 4.1, spot 26) is also down regulated in the IPC hearts. These results suggest that the ETC may be slowed down during IPC at 15 min reperfusion with subsequent detrimental consequences for the post-ischaemic hearts. A decreased electron transfer efficiency resulting from a decreased expression of complexes I and III promotes the formation of ROS [Cadenas et al., 1977]. It is well documented that oxidative stress plays a key role in heart disease. Mitochondria is a major source and target of reactive oxygen species (ROS) in the heart, due to the fact that it is the principle site of substrate oxidation in cardiomyocytes [Major et al., 2006]. ROS can damage cellular DNA, mitochondrial and cytosolic proteins, lipids and contractile apparatus thereby disrupting their normal structure and function. These results can explain why the IPC hearts are metabolically worse off compared to the Non-IPC hearts.

#### **4.7.6) Long chain specific acyl CoA dehydrogenase**

Acyl CoA dehydrogenases play a role in  $\beta$ -oxidation of fatty acids in the mitochondria as discussed in section 4.2. In the present study, the expression of long chain specific acyl CoA dehydrogenase (Table 4.1, spot 21) is increased in the IPC hearts. Interestingly, the expression of fatty acid binding protein (Table 4.1, spot 9), responsible for the transport of long chain fatty acids in the cell, is down regulated in the IPC hearts. This suggests that fatty acids in the IPC hearts accumulate in the cytosol because uptake into the mitochondria is retarded. Alternatively, down regulation of fatty acid binding protein in the IPC hearts suggests that fatty acids are transported by a different mechanism than in the Non-IPC hearts. In addition, the enhanced  $\beta$ -oxidation of fatty acids in the IPC

hearts could be responsible for the down regulated expression of glycolytic proteins. It is well established that enhanced fatty acid oxidation during reperfusion is detrimental to the ischaemic heart. Therefore the increased expression of long chain specific acyl CoA dehydrogenase in the IPC hearts compared to the Non-IPC hearts in the present study, can explain the poor functional recovery of the IPC hearts.

#### **4.7.7) Phosphatidylethanolamine binding protein (PEBP)**

Phosphatidylethanolamine binding protein (PEBP), also known as Raf kinase inhibitory protein, is a cytosolic protein that negatively regulates the mammalian mitogen activated protein kinases (MAPKs) [Wilsbacher & Cobb, 2001; Yeung et al., 1999]. PEBP inhibits Raf kinase by binding directly to Raf-1, MEK-1 and ERK-2, leading to inhibition and inactivation of Raf-1 with subsequent inhibition of the  $\beta$ -adrenergic receptor [Granovsky et al., 2009]. As a result, downstream MAPK signaling is interrupted and attenuated [Keller, 2011]. Expression of PEBP (Table 4.1, spot 11) is increased in the IPC hearts compared to Non-IPC. The ability of PEBP to negatively regulate Raf/MAPK signaling, could explain why activation of ERK is downregulated in the IPC hearts compare to the Non-IPC hearts at 15 min reperfusion (Results section, Chapter 3).

#### **4.7.8) Protein DJ-1**

Protein DJ-1 was discovered in 1997 as a novel oncogenic product [Nagakubo et al., 1997]. Emerging evidence indicate that this ubiquitously expressed protein has multiple cellular functions. DJ-1 plays an important role in the oxidative stress response by oxidizing itself to a more acidic form, thereby inducing the production of glutathione synthesis through an increase in glutamate ligase [Zhou & Freed, 2005]. Studies demonstrated that over-expression of DJ-1 protects against oxidative stress induced injury, whereas knockout of DJ-1 increases the susceptibility to oxidative injury [McCoy & Cookson, 2011]. DJ-1 deficient cells have higher levels of ROS, dysregulation of mitochondrial function, increased fragmentation and altered autophagy. It has been shown that mitochondrial fusion followed by fission events are a protective mechanism against oxidative stress. DJ-1 deficient cells have lower rates of mitochondrial fusion. A recent

study reported that DJ-1 is upregulated in hypoxic preconditioning through the activation of ERK p44/p42 [Lu et al., 2012].

In the present study, the expression of protein DJ-1 (Table 4.1, spot 36) is down regulated in IPC compare to Non-IP. It could possibly be due to the increased levels of ROS production in IPC, because of the inhibition of the ETC by the down regulation of the four electron carriers (Complex I, III, IV and electron transfer flavoprotein alpha). It is well documented that oxidative stress play a key role in heart disease [Costa et al., 2009], as discussed on page 157. The decreased expression of DJ-1 in the IPC hearts in the present study can therefore explain their poor functional recovery.

#### **4.7.9) Heat shock protein-beta-6**

Heat shock-beta-6, also known as HSP-20, is a member of the small heat shock protein family. HSP-20 is an inducible chaperone protein produced by cells in response to environmental changes and are expressed in skeletal muscle along with other heat shock proteins including alpha B crystalline and HSP-70 [Golenhofen et al., 2004]. HSP-27 and HSP-20 localize from the sarcoplasm to the myofibrils within 30 min after the onset of psychological stress [Golenhofen et al., 2004; Huot et al., 1996]. HSP-20 produces a similar response in cardiac muscle [Boluyt et al., 2006]. It is well documented that these proteins which include HSP-27, HSP-20, HSP-70 and alpha B crystallin are used as markers of pre-slaughter stress in a variety of animals [Morgan et al., poster - Carne Technologies, New Zealand; Boluyt et al., 2006]. The expression of HSP-20 (Table 4.1, spot 15) is up regulated in the IPC hearts, compared to the Non-IPC hearts, suggesting that the animals in the IPC group experienced pre-slaughter stress before the onset of the experiment. A proteomic study utilizing 2D analysis followed by MALDI-TOF mass spectrometry, demonstrated that the expression of HSP-20 was increased in an experimental model of heart failure [Cieniewski-Bernard et al., 2008]. Chronic heart failure was induced in male wistar rats by left coronary occlusion. The occlusion was permanent and no reperfusion occurred. Increased expression of HSP-20 in this study was associated with left ventricular remodeling (LVR), a process that occurs in response to myocardial damage [Pfeffer & Braunwald, 1990]. During this process the left ventricle undergoes



pathological changes when faced with an acute loss of contractile function [Yousef et al., 2002]. These results can explain why an increased expression of HSP-20 in the IPC hearts in the present study, may contribute to their poor functional recovery.

#### **4.7.10) Heat shock protein-60**

Heat shock proteins (HSP) are highly conserved proteins that play important roles in the cellular response to stress. HSP may enhance the survival rate of stressed cells by increasing the tolerance to stress [Iwaki et al., 1993; Fehrenbach et al., 2003]. HSP are also important role players in the emergence and development of cardiovascular disease [Snoeckx et al., 2001]. Studies demonstrated that HSP-60 localizes in the mitochondria where it has a crucial role in folding, unfolding and translocation of mitochondrial and cytosolic proteins. [Bukau & Horwich, 1998; Fink, 1999]. Interestingly, expression of HSP-60 (Table 4.1, spot 8) was down regulated in the present study in the IPC hearts compare to Non-IPC hearts. HSP-60 is also associated with psychological stress/trauma in animals and associated with transport stress, crowding and pre-slaughter stress. [Zhu et al., 2009; Bakau & Horwich, 1998]. Therefore, it may be possible that the animals in the Non-IPC group experienced psychological stress before the onset of the experiment. Since HSP-60 increases the survival rate of stressed cells by increasing the tolerance to stress, the decreased expression of HSP-60 in the IPC hearts however, can explain why these hearts showed poor functional recovery during reperfusion. In addition, HSP-60 has a crucial function in folding, unfolding and translocation of newly synthesized cytosolic and mitochondrial proteins. Downregulation of HSP-60 in the IPC hearts, suggests that these crucial functions are attenuated in these hearts and may contribute to poor recovery.

#### **4.7.11) Enoyl CoA hydratase**

Enoyl CoA hydratase triggers the metabolic breakdown of the following individual amino-acids; the branched chain amino-acids (Isoleucine, Valine and Leucine) and also Lysine. Degradation of Valine and Isoleucine by Enoyl CoA hydrates lead to the formation of Succinyl-CoA, which is converted to glucose via gluconeogenesis under the influence of glucocorticoids. Metabolic breakdown of



Leucine and Lysine by Enoyl CoA hydratase result in the formation of acetoacetate, which can be converted to fatty acid biosynthesis in the cytosol. In the present study Enoyl CoA hydratase (spot 19, Table 4.1) is upregulated in the IPC hearts compared to the Non-IPC hearts, suggesting that the IPC hearts are energy depleted (starved) and as a result non-carbohydrates (both amino-acids and acetoacetate) are used as fuel to synthesize glucose and fatty acids respectively. These results can explain why the IPC hearts in this part of the present study showed poor functional recovery.

#### **4.7.12) Transitional endoplasmic reticulum ATPase (TER ATPase)**

Transitional endoplasmic reticulum ATPase is a member of the ATPase family [Ye et al., 2001]. It is a cytosolic protein bound to ER membranes that binds and transports ubiquitinated proteins from the ER to the cytosol, where they are degraded by the proteasome. This is an ATP dependent process, as TER ATPase uses ATP hydrolysis to perform this function. Its major function is proteasome mediated protein degradation, but TER ATPase is also involved in a wide variety of cellular activities. These include membrane fusion, vesicle transport and dissociation of an ubiquitinated membrane transcription factor from its non-ubiquitinated binding partner. It prevents excessive DNA synthesis and limit the incidence of mutations induced by DNA damage [Ye et al., 2001].

Stimulation of the G $\alpha$ q-protein-coupled receptors (GPCR's) activates phospholipase C resulting in the formation of the 2<sup>nd</sup> messenger inositol 1,4,5-triphosphate (IP<sub>3</sub>) and Diacylglycerol (DAG). IP<sub>3</sub> diffuses into the cytosol and bind to the IP<sub>3</sub> receptors located in the membranes of the ER, thereby releasing calcium from this organelle. IP<sub>3</sub> receptors play therefore a pivotal role in converting extracellular stimuli into intracellular calcium signals. In response to hormonal stimulation of GPCR's that persistently elevate the concentration of IP<sub>3</sub>, the cellular level of IP<sub>3</sub> are rapidly reduced. This process is known as IP<sub>3</sub> downregulation and has been shown in various mammalian tissues.

Downregulation of IP<sub>3</sub> receptors is a protective mechanism that protects cells against the harmful effects of elevated cytosolic calcium levels as a result of

alpha-GPCR stimulation. IP<sub>3</sub>R downregulation is mediated by the ubiquitin-proteasome pathway (UPP) and is a form of endoplasmic reticulum associated degradation [Alzayady et al., 2005]. TER ATPase associates with IP<sub>3</sub> receptor in response to hormonal stimulation that induce IP<sub>3</sub> receptor ubiquitination. TER ATPase is therefore a protective mechanism that protects cells against the deleterious effects of chronic elevated calcium levels [Alzayady et al., 2005].

In the present study TER ATPase (spot 22, Table 4.1), was downregulated in the IPC hearts compared to the Non-IPC hearts, suggesting the Non-IPC hearts are protected against calcium overload during reperfusion. These results suggest that downregulation of IP<sub>3</sub> by TER ATPase in the IPC hearts was suppressed compared to Non-IPC. In addition calcium levels might have been elevated in IPC during reperfusion and this can explain why the IPC hearts showed poor functional recovery.

#### **4.7.13) 78 kDa Glucose regulated protein (GRP78)**

Mammalian cells respond to glucose deprivation by synthesizing a set of proteins known as the glucose-regulated proteins, GRPs [Shiu et al., 1997]. GRP78 is an endoplasmic reticulum protein and a central regulator of ER function. It plays an important role in protein folding and assembly, ER calcium binding and the targeting of misfolded proteins for degradation [Chang et al., 1987]. Apart from its role as an ER chaperone, GRP78 also functions as an anti-apoptotic protein [Zhou et al., 2011]. BCL-2-interacting killer (BIK) is a BH3 only protein and a pro-apoptotic tumor suppressor. Its function is regulated at the ER where endogenous BIK forms selectively complexes with GRP78, but not with other ER chaperones [Zhou et al., 2011]. BIK rely on its BH3 domain to interact with GRP78. Overexpression of GRP78 blocks BIK-induced apoptosis by inhibiting the release of calcium from the ER stores; Bax translocation to the mitochondria, the release of cytochrome c into the cytosol, activation of caspases, mitochondrial permeability transition and consequent apoptosis [Chang et al., 1987].

In the present study, GRP78 (spot 23, Table 4.1) is downregulated in the IPC hearts compared to the Non-IPC hearts. This may suggest that the IPC hearts

are undergoing apoptosis and can explain why they showed poor functional recovery.

#### **4.7.14) Protein disulfide isomerase A3 (PDI-A3)**

PDI-A3 is a member of the PDI family of proteins of the endoplasmic reticulum and is activated in response to cellular stress [Ni & Lee, 2007]. Its expression increases following glucose depletion and it also has a role in calcium regulation [Appenzeller-Herzog & Ellgaard, 2008; Turano et al., 2011]. Oxidative stress and dysregulation of calcium play an important role in heart failure [Afanas'ev, 2011]. It is well known that the NADPH oxidases (Nox), xanthine oxidase, mitochondria and nitric oxide synthetases (NOS) are sources of ROS and RNS [Afanas'ev, 2011]. Nox4 increases the mitochondrial level of superoxide, resulting in mitochondrial and LV dysfunction [Afanas'ev, 2011]. Calcium leak from the SR and decreased blood flow are associated with increased production of superoxide radicals in HF. Both oxidative and hypoxic stresses have been shown to increase the expression of PDI-A3 [Toldo et al., 2011]. Increased levels of PDI-A3 is associated with HF in both human and animal models. This was linked to calcium dysregulation and apoptosis, suggesting a role for PDI-A3 in the progression of heart disease [Vitello et al., 2012]. In the present study PDI-A3 (spot 31, Table 4.1) is upregulated in the IPC compared to the Non-IPC hearts. These results suggest that ROS and calcium levels may be increased in the IPC hearts with detrimental consequences, resulting in poor recovery.

#### **4.8) Discussion: Mitochondrial proteomics**

Some of the differentially regulated proteins (Table 4.2) will be individually, discussed in this section.

#### **4.8.1) Long chain specific acyl CoA dehydrogenase**

Acyl CoA dehydrogenases play a role in  $\beta$ -oxidation of fatty acids in the mitochondria as discussed in section 4.2. In the present study, the expression of long chain specific acyl CoA dehydrogenase (Table 4.2, spot 3) is increased in the IPC hearts. As mentioned before, the expression of fatty acid binding protein (Table 4.1, spot 9), responsible for the transport of long chain fatty acids in the cell, is down regulated in the IPC hearts. This suggests that fatty acids in the IPC hearts accumulate in the cytosol because uptake into the mitochondria is retarded. Alternatively, down regulation of fatty acid binding protein in the IPC hearts suggests that fatty acids are transported by a different mechanism than in the Non-IPC hearts. In addition, the enhanced  $\beta$ -oxidation of fatty acids in the IPC hearts could be responsible for the down regulated expression of glycolytic proteins. It is well established that enhanced fatty acid oxidation during reperfusion is detrimental for the ischaemic heart. Therefore the increased expression of long chain specific acyl CoA dehydrogenase in the IPC hearts compared to the Non-IPC hearts in the present study, can explain the poor functional recovery of the IPC hearts.

#### **4.8.2) Complex 1 of the Electron Transfer Chain (ETC)**

Mitochondria contain a series of catalysts known as the electron transport chain (ETC) that collects and transports reducing equivalents in a final common pathway to direct them in a final reaction with oxygen leading to the formation of water. These catalysts comprises of NAD-linked dehydrogenase systems, flavoproteins and cytochromes. Also present in the ETC is the machinery responsible for trapping the generated free energy as high energy phosphate.

Ubiquinone or Q (also known as Complex I or coenzyme Q) is an additional carrier present in the ETC linking the flavoproteins to cytochrome b. Q exists in mitochondria in the oxidized quinone form under aerobic conditions and in the reduced quinol form under anaerobic conditions. Q serves as the entry point of electrons into the ETC and pass them on to complex III and IV. In the present study, expression of Complex 1 or Q (Table 4.2; spot 1) of the ETC is down regulated in the IPC hearts. Since Complex 1 serves as the entry point of

electrons to the ETC, these results suggest that the ETC may be retarded during IPC at 15 min reperfusion, with subsequent detrimental consequences for the post-ischaemic hearts.

#### **4.8.3) Complex V (ATP Synthase)**

ATP synthase or complex V of the ETC, also known as the proton pumping synthase is the most complex structure in the mitochondrial inner membrane. It facilitates proton flux to drive ATP synthesis. The endergonic synthesis of ATP from ADP and Pi is catalyzed by ATP synthase, which is driven by the electron transport process through complexes I, II and IV. A proteomic study utilizing the 2D-DIGE technique followed by MALDI-TOF mass spectrometry demonstrated that expression of ATP synthase was reduced in the spontaneous hypertensive rat, which confirmed the deprivation of ATP production in the very early stages of left ventricular hypertrophy before hypertension [Meng et al., 2005]. In the present study ATP synthase (spot 6, Table 4.2) is downregulated in the IPC hearts compared to the Non-IPC hearts. These results suggest that the ETC is not functioning in the IPC hearts, since Complex I, II and IV are also downregulated in the IPC hearts. Since the generation of ATP by the ETC is crucial for pump function and functioning of the ion channels, these results can explain why the IPC hearts showed poor functional recovery in the present study.

#### **4.8.4) Mitofilin**

Mitofilin exists as a large protein complex in the narrow space between the inner boundary and the outer mitochondrial membrane where it controls mitochondrial cristae morphology. Downregulation of mitofilin was shown to be associated with increased apoptosis and abnormal mitochondrial function [John et al., 2005]. 2D analysis of mitochondrial proteins in a Langendorff model, using rabbit hearts demonstrated that mitofilin was downregulated in IPC hearts compared to Non-IPC hearts, after 60 min reperfusion [Kim et al., 2006]. These results support the results of the present study in which mitofilin (spot 20, table 4.2) was downregulated in the IPC hearts compared to the Non-IPC hearts. The increased expression of mitofilin in the Non-IPC hearts can be explained as a protective

recovery mechanism against ischaemia/reperfusion injury of the mitochondria [Kim et al., 2006]. Increased expression of mitofilin is associated with maintenance of normal mitochondrial function during stress and protective against the collapse of main mitochondrial function. This can offer a possible explanation why the Non-IPC hearts (with an increased expression of mitofilin) in the present study are metabolically better off than the IPC hearts.

#### **4.8.5) Heat shock protein 60 (HSP-60)**

Heat shock proteins (HSP) are highly conserved proteins that play important roles in the cellular response to stress. HSP may enhance the survival rate of stressed cells by increasing the tolerance to stress [Iwaki et al., 1993; Fehrenbach et al., 2003]. HSP are also important role players in the emergence and development of cardiovascular disease [Snoeckx et al., 2001]. It was demonstrated that HSP-60 localizes in the mitochondria where it has a crucial role in folding, unfolding and translocation of mitochondrial and cytosolic proteins. [Bukau & Horwich, 1998; Fink, 1999]. A proteomic study utilizing 2D analysis, followed by MALDI-TOF mass spectrometry demonstrated that HSP-60 was upregulated in cardiomyocytes by sustained levels of adrenalin [Costa et al., 2009]. The same study showed that when cardiomyocytes were subjected to a combination of sustained levels of adrenalin and increased levels of ROS, HSP-60 expression was decreased. The protocol did not include any reperfusion [Costa et al., 2009] Interestingly, expression of HSP-60 (Table 4.2, spot 19) was down regulated in the present study in the IPC hearts compare to Non-IPC hearts. HSP-60 is also associated with psychological stress/trauma in animals and associated with transport stress, crowding and pre-slaughter stress. [Zhu et al., 2009; Bakau & Horwich, 1998]. Therefore, it may be possible that in the present study the animals in both groups (IPC and Non-IPC) experienced phsycological stress before the onset of the experiment. Since HSP-60 increases the survival rate of stressed cells by increasing the tolerance to stress, the decreased expression of HSP-60 in the IPC hearts however, can explain why these hearts showed poor functional recovery during reperfusion.

#### **4.8.6) Creatine Kinase M-type (mitochondrial type)**

In cardiac muscle the enzyme creatine kinase transports high energy phosphates between ATP and creatine. It forms part of a creatine phosphate shuttle that transports high energy phosphates from the mitochondria to the sarcolemma. In the myocardium this shuttle may be important in affording immediate protection against the effects of infarction. A proteomic study utilizing 2D analysis, followed by MALDI-TOF mass spectrometry, demonstrated that the expression of creatine kinase M-type was increased in cardiomyocytes subjected to sustained elevated levels of adrenaline. This was accompanied by decreased expression of glycolytic enzymes and the protective HSP-60, HSP-70 and Alpha  $\beta$ -Crystallin [Costa et al., 2009]. The protocol did not include any reperfusion. In the present study the expression of creatine kinase was increased in the IPC hearts (spot 15, Table 4.2) compared to the Non-IPC hearts.

#### **4.8.7) Aconitase**

Mitochondrial Aconitate hydratase (Aconitase) catalyzes the reaction of reversible isomerization of citrate to isocitrate. This reaction is the initiating step of the TCA cycle. Aconitase bind the mitochondrial chaperones HSP-70, HSP-60 and HSP-40, very specifically, resulting in the formation of a stable complex [Matasova & Poppova, 2008]. The function of Aconitase is mainly to regulate citrate accumulation and to direct it to lipogenesis. There upon it affects the level of free fatty acids. Aconitase also functions as a neurotransmitter. Aconitase is very sensitive to ROS and can influence the formation of ROS under conditions of oxidative stress, such as ischaemia in the following ways: (1) inhibition of the initiating stage of the TCA (2) change the reduction of the carriers of the mitochondrial respiratory chain, (3) influence the intensity of lipogenesis and the pool of free fatty acids and as a result provide uncoupling of respiration and phosphorylation [Matasova & Popova]. A proteomic study utilizing 2D-DIGE followed by MALDI -TOF mass spectrometry demonstrated that Aconitase was upregulated in spontaneous hypertensive rats. This study showed that the increased expression of Aconitase contributed to the development of cardiac hypertrophy [Meng et al., 2009]. A different proteomic study utilizing 2D



proteomics, followed by MALDI-TOF mass spectrometry, showed that the expression of Aconitase was upregulated in cardiomyocytes subjected to sustained elevated levels of adrenalin. The protocol did not include any reperfusion [Costa et al., 2009]. The sustained levels of catecholamines and ROS are well recognized hallmarks of several pathological conditions like ischaemia/reperfusion injury and heart failure [Behonick et al., 2001; Bindoli et al., 1992]. In this particular study the increased expression of Aconitase was associated with pathological levels of adrenaline [Costa et al., 2009]. In the present study Aconitase is upregulated in the IPC hearts (spot 25, Table 4.2) compared to the Non-IPC hearts. These adverse effects of increased expression of Aconitase can explain why the majority of ETC carriers are downregulated in the IPC hearts. It can also explain the increase in generation of free fatty acids in the IPC hearts, with the subsequent decrease in glycolytic enzymes. The increase in fatty acids can lead to uncoupling of respiration from oxidative phosphorylation in the IPC hearts. Taken together this can explain why the IPC hearts are comprimized at a metabolic level.

#### **4.8.8) Heat shock protein-70 (HSP-70)**

HSP-70 is a member of the heat shock protein family as discussed and serves as a molecular chaperone. It plays a major role in protecting cells against different cellular stressors, such as heat, hypoxia, oxidative stress, ultraviolet irradiation, apoptosis and necrosis [Zhang et al., 2012]. Despite its protective functions, HSP-70 is also used as a marker of pre-slaughter stress in animals [Bao et al., 2008; 2002; Cvorovic et al., 1998]. A proteomic study utilizing 2D-DIGE followed by MALDI-TOF mass spectrometry demonstrated that HSP-70 was upregulated in 34 patients suffering from chronic heart failure [Wei et al., 2009]. This increased expression of HSP-70 in the cardiac tissue was accompanied by an elevation of circulating HSP-70 in heart failure patients. These patients suffer from the following cardiomyopathies; arrhythmogenic right ventricular cardiomyopathy (ARVC), dilated cardiomyopathy (DCM) and ischaemic cardiomyopathy (ICM). A different proteomic study utilizing 2D proteomics followed by MALDI-TOF mass spectrometry, demonstrated that HSP-70 was downregulated in cardiomyocytes subjected to sustained pathological levels of adrenaline [Costa et al., 2009]. The protocol did not include reperfusion [Costa et al., 2009]. In the present study,



HSP-70 expression (Table 4.2, spot 7) was down regulated in IPC compared to Non-IPC. Since the present study was carried out with normal healthy animals these results suggest that the animals in both groups (IPC and Non-IPC) experienced psychological stress before the onset of the experiment. The Non-IPC hearts had the ability to induce a protective strategy by increasing the expression of the protective HSP-70 family of proteins. The IPC hearts, on the other hand were unable to increase the expression of the protective HSP-70 family of proteins, possibly due to the increased formation of ROS as a result of the multiple episodes of ischaemia. These results can explain the poor recovery of the IPC hearts.

#### **4.8.9) Alpha $\beta$ -Crystallin**

Alpha  $\beta$ -crystallin is a member of the small heat shock protein family that functions as chaperones contributing to cell survival. It also acts as an anti-oxidant and free radical scavenger. The expression levels of alpha  $\beta$ -crystallin are high in cardiac muscle. It comprises 3% of total cardiac protein content. It is well documented that the heat shock protein family are upregulated in response to psychological stress. A proteomics study, utilizing 2D analysis combined with MALDI-TOF mass spectrometry demonstrated increased expression of alpha  $\beta$ -Crystallin in the presence of sustained levels of adrenaline in a cardiomyocyte model without reperfusion [Costa et al., 2009]. In the same study the expression of alpha  $\beta$ -Crystallin was decreased when sustained levels of adrenaline was combined with increased levels of ROS [Costa et al., 2009]. The expression of alpha  $\beta$ -crystalline (Table 4.2, spot 17) is upregulated in Non-IPC compared to IPC. The pro-survival functions of alpha  $\beta$ -crystalline mentioned above can explain why the Non-IPC hearts are metabolically advantaged compared to the IPC hearts. Interestingly, alpha  $\beta$ -crystallin was shown to be activated by p38 MAPK [Aggeli et al., 2008] and in the present study, p38 MAPK was significantly activated in Non-IPC compared to IPC at 15 min reperfusion. This finding [Aggeli et al., 2008] therefore supports our results.

#### **4.8.10) Short Chain Specific Acyl-CoA dehydrogenase**

Fatty acids can be metabolized by different mechanisms. Long chain fatty acids

are degraded by  $\beta$ -oxidation in the mitochondria, while short chain fatty acids are degraded by omega oxidation. It is normally a very minor pathway and involves P-450 in the endoplasmic reticulum [Wanders et al., 2010]. Short chain specific acyl-CoA dehydrogenases play a role in this process. Short chain specific acyl CoA is upregulated in IPC hearts (Table 4.2, spot 9), compared to Non-IPC, suggesting that fatty acids are metabolized by omega-oxidation in IPC.  $\beta$ -oxidation is the preferred pathway for the metabolism of fatty acids and yield more high energy phosphates, but omega oxidation becomes important when  $\beta$ -oxidation in the mitochondria is impaired [Wanders et al., 2010], as seen in IPC in the present study. These results can explain why the IPC hearts are metabolically disadvantaged compared to the Non-IPC hearts in the present study.

#### **4.8.11) Aldehyde dehydrogenase**

The expression of aldehyde dehydrogenase (Table 4.2, spot 18) is up regulated in IPC compared to Non-IPC. Aldehyde dehydrogenase is also involved in omega oxidation of fatty acids in the endoplasmic reticulum [Nelson & Cox, 2005], in light of the above mentioned, this result supports our earlier finding that beta oxidation is compromised in the IPC hearts, resulting in the accumulation of fatty acids during reperfusion. These results can explain why the IPC hearts are metabolically compromised in the present study.

#### **4.8.12) Malate dehydrogenase**

Cytosolic malate dehydrogenase is involved in gluconeogenesis by catalyzing the production of oxaloacetate from malate. Malate is produced from oxaloacetate in the mitochondria and diffuses from the mitochondria into the cytosol where it is converted back to oxaloacetate, leading to the formation of phosphoenolpyruvate. Phosphoenolpyruvate is involved in the reversal of glycolysis resulting in the production of glucose. This process forms part of the gluconeogenesis pathway, whereby non-carbohydrates are converted to glucose under energy deprived situations when glycolysis is inhibited.

A proteomic study utilizing 2D-DIGE followed by MALDI -TOF mass spectrometry demonstrated that Malate dehydrogenase was upregulated in spontaneous hypertensive rats. This study showed that the increased expression of Malate dehydrogenase contributed to the development of cardiac hypertrophy [Meng et al., 2009]. A different proteomic study utilizing 2D analysis combined with MALDI-TOF mass spectrometry demonstrated that expression of Malate dehydrogenase was upregulated in myocytes subjected to sustained elevated levels of adrenaline. The protocol did not include reperfusion [Costa et al., 2009]. In the present study, expression of malate dehydrogenase (Table 4.1, spot 16) was upregulated in IPC compared to Non-IPC, suggesting that IPC utilized the gluconeogenic pathway in order to replenish the shortage of glucose. Since gluconeogenesis is the preferred metabolic pathway in the IPC hearts instead of glycolysis, this can explain why the IPC hearts are metabolically disadvantaged in the present study. These results also suggest that animals in both groups (IPC and Non-IPC) experienced psychological stress, but the Non-IPC hearts could adapt by upregulation of the glycolytic enzymes.

#### **4.8.13) Nucleoside Diphosphate kinase**

Nucleoside Diphosphate kinase (NDPK) catalyzes the non-substrate specific conversion of nucleoside diphosphates to nucleoside triphosphates [Zhou & Artman, 2001]. It has been found in the hearts of many species, where it participates in a wide range of biological functions including gene regulation, development, growth and signal transduction [Heidbuchel et al., 1992]. The expression and activation of NDPK in the heart is regulated by  $\beta$ -adrenergic signaling [Lutz et al., 2003]. NDPK catalyzes the transfer of terminal phosphate groups from 5'-triphosphate to 5'-diphosphate nucleotides. The main reaction is the synthesis of GTP from ATP, to maintain levels of GTP, required for activation of G proteins [Otero 1990; Kimura 1993; Piacentini & Niroomand 1996]. A study by Niroomand and his group demonstrated that both expression levels and activation of NDPK are significantly increased in failing human hearts compared to normal non-failing controls [Lutz et al., 2001]. Patients treated with  $\beta$ -blockers who underwent cardiac surgery had a 65% lower DNPK content compared to those who had no  $\beta$ -blocker therapy [Lutz et al., 2001]. The lower levels of DNPK were associated with a reduced DNPK- dependent Gi-mediated inhibition of

adenylyl cyclase activity. Rats treated with a  $\beta$ -adrenergic agonist, isoproterenol developed myocardial hypertrophy. This was accompanied by an increase in DNPK levels by 60%. Treatment with the  $\beta$ -blocker propranolol prevented the development of cardiac hypertrophy and DNPK levels did not rise [Lutz et al., 2001]. Taken together, these results demonstrated that chronic stimulation of the  $\beta$ -adrenergic receptors increase DNPK levels. This pathway provides a mechanism that compensates in part for the downregulation of  $\beta$ -receptors that paralleled chronic  $\beta$ -stimulation in order to modulate cAMP levels independent of receptor-G-protein interaction [Zhou & Artman, 2001]. In the present study, the expression of DNPK (spot 17, Table 4.2) was upregulated in the IPC hearts compared to the Non-IPC hearts. This finding may suggest that the IPC hearts underwent chronic  $\beta$ -stimulation followed by desensitization of the  $\beta$ -receptors. This can explain the poor functional recovery (contractile function) of the IPC hearts.

#### **4.8.14) Generation of amino acids**

The expression of proteins involved in the metabolic breakdown of amino acids was up regulated in IPC compare to Non-IPC. Expression of dihydrolipoyl dehydrogenase (Table 4.2, spot 5) is up regulated in IPC and similarly the expression of mercaptopyruvate sulfurtransferase (Table 4.2, spot 10). Amino acids can fuel the Krebs cycle by entering the cycle at several entry points and thereby enhance glucose biosynthesis via gluconeogenesis as a result of glucocorticoid secretion. This supports the rest of the data which demonstrate that glycolysis is attenuated in the IPC hearts and can explain why the IPC hearts is metabolically disadvantaged in the present study.

#### **4.8.15) Annexin A3**

The annexins are a family of proteins which are involved in a variety of cellular processes, including inflammation, apoptosis, membrane fusion, proliferation and differentiation [Gerke et al., 2002]. Annexin A3 is a physiological regulator of angiogenesis [Park et al., 2005]. Angiogenesis is the process whereby new blood vessels are generated from existing ones. It occurs under normal, but also

pathological conditions. Physiological angiogenesis is critical for wound healing, development and reproduction [Park et al., 2005]. Studies demonstrated that annexin A3 induces the migration and tube formation of human umbilical vein endothelial cells. In addition, expression of annexin A3 activates HIF-1 activity, suggesting that annexin A3 promotes up regulation of the HIF-1 signaling pathway which is also involved in angiogenesis [Park et al., 2005]. HIF-1 prevents cardiac and tissue damage as a result of ischaemia by inducing angiogenesis thus increasing oxygen delivery to the area. In the present study, expression of annexin A3 (Table 4.2, spot 13] is increased in Non-IPC compare to IPC, suggesting that oxygen delivery to the post-ischaemic area may be increased in Non-IPC hearts.

**Taken together**, the results from the cytosolic and mitochondrial data generated in the present study suggest the following modifications of the metabolism in the IPC hearts: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is a key energy regulator of glycolysis in the cardiomyocyte is down regulated in the IPC hearts (Table 4.1, spot 18). This enzyme is normally down regulated during ischaemia since ischaemia uncouples glycolysis from oxidative phosphorylation [Opie, 1998]. This is due to the low oxygen levels that suppress the ETC, which in turn inhibits the transfer of NADH and  $H^+$  to the mitochondria. Accumulation of NADH and  $H^+$  in the cytosol inhibits GAPDH and thus glycolysis [Opie, 1998]. Removal of NADH from the cytosol to the mitochondria during early reperfusion is thus essential to maintain the levels of GAPDH and thus sustain glycolysis. Transfer of NADH from the cytosol to the mitochondria is normally facilitated by lactate dehydrogenase (LDH), which is down regulated (Table 4.1, spot 28) in the IPC hearts. As a result NADH will accumulate in the cytosol, which inhibits LDH further and thus GAPDH and glycolysis, resulting in damage to the myocardium [Neely & Grotyohan, 1984]. The protective mechanism of LDH mediated removal of NADH is sometimes limited as a result of poor waste removal when the myocardium is inadequately reperfused. Since this study was carried out at 15 min reperfusion, it is expected that waste removal (lactate accumulated during ischaemia), in the now perfused myocardium of the IPC hearts should have been sufficient. In addition, lactate inhibits PDH and inhibits glucose uptake [Depré, Rider & Hue, 1998]. Therefore, different substrates (fatty acids, amino acids and lactate) are utilized by the IPC hearts as an alternative source of energy. In

addition, down regulation of PDH may switch pyruvate metabolism towards oxaloacetate in order to replenish the Krebs cycle intermediates and switch the cycle towards glucose biosynthesis via gluconeogenesis as a result of glucocorticoid (corticosterone) stimulation [Stanley et al., 1997; Taegtmeyer 1994].

Another factor that can contribute or might be responsible for the down regulation of glycolysis in the IPC hearts is the upregulation of the oxidation of fatty acids. The fatty acid transporter is downregulated in the IPC hearts, suggesting that fatty acids may accumulate in the cytosol. Since it is generally accepted that fatty acids decrease cardiac efficiency [Challoner & Steinberg, 1966], the build-up of fatty acids in the cytosol may be detrimental to the IPC hearts.

Studies in the isolated working heart demonstrated that fatty acid oxidation inhibits glycolysis to a greater extent than what glycolysis can inhibit fatty acid oxidation; however, the heart functions best when it oxidizes two substrates simultaneously [Taegtmeyer et al., 1980]. It is generally accepted that fatty acids decrease cardiac efficiency [Challoner & Steinberg, 1966]. The main reason for contractile dysfunction is thought to be the delayed clearance of protons from the cell. One hypothesis is that increased glucose oxidation during reperfusion reduces the proton load and improves functional recovery [Challoner & Steinberg, 1966; Burkhoff et al., 1991].

In the present study, four mitochondrial proteins associated with electron transfer in the ETC are down regulated in the IPC hearts, suggesting that the ETC and oxidative phosphorylation may be attenuated. Oxidative phosphorylation in the mitochondria is the main source of ATP generated for cardiac contractile function [Bugger et al., 2010]. It is well documented that impairment of mitochondrial function contributes to mechanical dysfunction in numerous cardiac disease states [Bugger et al., 2009; Rosca et al., 2008; Lesnefsky et al., 2001]. These results could offer a possible explanation of the underlying causes (mechanisms involved) or factors contributing to poor functional recovery of the IPC hearts after 15 min reperfusion.

In addition, results from this study demonstrated that the gluconeogenesis pathway is upregulated in IPC compare to Non-IPC. Gluconeogenesis is stimulated by the glucocorticoids, which is known to inhibit glycolysis. It is well documented that the production of corticosterone is enhanced in animals during stressfull events, such as crowding, transportation and pre-slaughter stress. Alpha  $\beta$ -crystallin, HSP-27, HSP- 60, HSP-70 associated with pre-slaughter stress are upregulated in Non-IPC and HSP-20 associated with pre-slaughter stress is upregulated in IPC. These results indicate that animals in both groups experienced psychological stress before the hearts were excised, but the Non-IPC could counteract the stress, as indicated by the unaltered metabolism.

Expression of NDPK was increased in the IPC hearts: This pathway provides a mechanism that compensates in part for the downregulation of  $\beta$ -receptors that paralleled chronic  $\beta$ -stimulation in order to modulate cAMP levels independent of receptor-G-protein interaction [Zhou & Artman, 2001]. The contractile proteins Troponin T and Myosin Light chain 3 were downregulated in the IPC hearts, supporting possible downregulation of the  $\beta$ -receptor.

Expression of anti-apoptotic proteins were increased in Non-IPC hearts compare to IPC hearts, suggesting that apoptosis was induced in IPC hearts.

#### **4.9) Conclusion**

Here we demonstrated the use of 2-DE proteomics for detection of significant differences in protein expression levels between IPC and Non-IPC hearts during early (15 min) reperfusion. It is generally believed that IPC is a  $\beta$ -receptor dependent process [Lochner et al., 1999; Kovanecz et al., 1996]. Previous studies supported these findings that stimulation with the  $\beta$ -agonists norepinephrine or isoproterenol mimics IPC [Asimakis et al., 1994]. Studies performed in our laboratory showed no evidence that the  $\alpha$ -adrenergic receptor or the PKC signal transduction pathway is involved in IPC-induced protection [Moolman et al., 1996].

Results from the present study however, suggest that animals in the IPC group experienced chronic  $\beta$ -stimulation, as a result of psychological stress, followed by



downregulation and desensitizing of the  $\beta$ -receptors. This resulted in signaling through  $\alpha$ -adrenergic receptors which was accompanied by suppression of contractility and a subsequent attenuation of MAPK signaling in IPC hearts. The  $\alpha$ -adrenergic receptor is a G-protein-coupled receptor (GPCR) associated with the Gq heterotrimeric G-protein. The catecholamines like norepinephrine (noradrenaline) and epinephrine (adrenaline), which are known  $\beta$ -receptor agonists also signal through the  $\alpha$ -adrenergic receptor. Upon activation of the  $\alpha$ -Gq receptor by norepinephrine and epinephrine the 2<sup>nd</sup> messenger inositol 1,4,5-triphosphate (IP<sub>3</sub>) and Diacylglycerol (DAG) is generated. IP<sub>3</sub> diffuses into the cytosol and bind to the IP<sub>3</sub> receptors located in the membranes of the ER, thereby releasing calcium from this organelle. Under normal conditions downregulation of the IP<sub>3</sub> receptor occur to protect the cell against the harmful elevated levels of calcium. The results from the present study indicate that downregulation of the IP<sub>3</sub> receptor failed, resulting in calcium overload in the IPC hearts.

In addition, results suggest that the ETC and oxidative phosphorylation may be attenuated. Oxidative phosphorylation in the mitochondria is the main source of ATP generated for cardiac contractile function [Bugger et al., 2010]. It is well documented that impairment of mitochondrial function contributes to mechanical dysfunction in numerous cardiac disease states [Bugger et al., 2009; Rosca et al., 2008; Lesnefsky et al., 2001]. Mitochondrial dysfunction gives rise to the accumulation of ROS and causes oxidative stress. As a result oxidative stress stimulates cardiac hypertrophy through a variety of hypertrophy signaling cascades and transcription factors [Takimoto & Kass 2007; Sabri et al., 2003]. Studies have shown that downregulation of complexes I, III, IV and V in mitochondria were the source of elevating damaging free radicals [Roche et al., 2009] and was associated with elevated blood pressure and decreased ATP synthesis [Lopez-Campistrous et al., 2008]. Studies have shown that the following factors, a decrease in cellular ATP levels, accumulation of hydrogen ions, calcium overload and the production of ROS, contribute to ischaemia/reperfusion injury [French et al., 2006; Zweier et al., 2006].

Taken together, the data from the present study suggest that the IPC hearts experienced extreme or even possibly irreversible ischaemia, whereas the Non-



IPC hearts experienced only mild and reversible ischaemia. During early ischaemia in animal models, there is a variable and possibly predominant element of reversibility with eventually irreversibility. The term ischaemia was first used by Rudolf Virchow in 1858. The concept was described as the limitation of blood supply with an increased resistance to flow. Myocardial ischaemia exists when the reduction of coronary flow is so severe that the oxygen supply to the myocardium is inadequate to meet the required demands of the cardiac tissue. Two phases of the adaptation of the oxygen deficient tissue are proposed: namely short-term defence and long term rescue [Hochachka et al., 1996]. The aim of short term defence is to achieve a new balance between oxygen demand and supply. This is achieved by a combination of a downregulation of contraction and upregulation of anaerobic energy production by glycolysis to compensate in part for oxidative metabolism. The aim of long term rescue is to induce a series of cellular signals that lead to protective genetic reprogramming. These long term adaptations may account for unexpected protective reactions to ischaemia, such as the synthesis of new proteins, as demonstrated in the Non-IPC hearts. The metabolic consequences of myocardial ischaemia can be divided into 2 major processes: 1) those caused by inadequate oxygen supply 2) and those caused by inadequate washout of the metabolites of ischaemia. Common to both processes is an increase of cytosolic calcium.

In animal models there is rapid recovery from ischaemia that is not accompanied by contracture. The period after potentially reversible ischaemia (early reperfusion) is associated with an intracellular accumulation of protons as well as sodium and calcium ions. Decreasing the proton load during early reperfusion by implementing sodium-proton exchange inhibitors have shown a more rapid recovery from ischaemia. These results from numerous studies suggest that operation of this exchanger help to normalize the cell's pH [Liu et al., 1996; Vandenberg 1993]. Several metabolic changes occur during recovery from ischaemia (early reperfusion); 1) Mitochondrial metabolism is depressed (as seen in the IPC group); 2) fatty acids appear to compete better than glucose for this residual metabolism (as seen in the IPC group); 3) Glycolysis is depressed (as seen in the IPC group). Glycolysis is beneficial and not harmful during reperfusion, since it supply energy for the calcium uptake pump of the sarcoplasmic reticulum, thereby decreasing the internal calcium load

accumulated during ischaemia [Jeremy, 1992]. A study by Sebbag and coworkers in 1996, indicated that a decrease in glycolysis markedly accelerated cell death in dogs after coronary occlusion. Thus a decrease of glycolysis during reperfusion is postulated to be a crucial factor in the progression of reversible ischaemia to ischaemic cell necrosis. The upregulation of glycolytic enzymes in the Non-IPC hearts in the present study suggest beneficial effects. The mechanisms whereby reversible ischaemic damage develops into irreversible infarction are: 1) loss of a critical amount of ATP; 2) membrane damage induced metabolically or mechanically; 3) formation of free radicals; 4) calcium overload and 5) sodium pump inhibition. Inhibition of the sodium pump may result in an excess of internal sodium, which in turn leads to an increase in osmotic pressure, which helps to rupture the cell membrane and to cause irreversible damage. The proposed cause of pump failure was an inadequate synthesis of sufficient glycolytic ATP for the functioning of the sodium pump [Cross, 1995]. Membrane damage includes 1) accumulation of free fatty acids inside and outside of ischaemic cells. Part of the membrane damage results from the action of phospholipases that break down membrane lipids. The resulting lysophosphoglycerides further promote membrane damage 2) lipid peroxide formation by oxygen free radicals. Free radicals are derived in part from neutrophils particularly during the reperfusion phase of ischaemic damage and in part from damaged cardiomyocyte mitochondria. The concept of calcium overload resulting in irreversible ischaemic damage has received special prominence in relation to conditions of massive calcium overload, such as catecholamine stimulation and severe reperfusion damage. This was illustrated by the experimental condition described as the calcium paradox. This entails the total removal of extracellular calcium after which calcium is re-introduced, causing massive cellular damage. The basic concept of calcium overload is that the mitochondria takes up calcium from the cytosol, thereby acting as a buffer. This process requires a considerable amount of energy. When energy depletion is enhanced, the energy required for maintenance of ionic gradients becomes inadequate and membrane integrity is lost. During ischaemia cytosolic calcium levels increase and this is accompanied by activation of phospholipases, increasing resting tension and promotion of fatal arrhythmias [Lubbe 1992]. Therefore, irreversible injury probably depends on no single metabolic event, but

may be a complex phenomenon resulting from the simultaneous operation of many diverse mechanisms.

Earlier studies identified emotional and physical stress as triggers in up to half of the cases of AMI in humans, as a result of increased adrenergic activation, originating from either intense arousal or severe physical exercise [Tofler, 1990]. Later studies revealed a link between psychological stress and sudden death, resulting from cardiac complications [Lampert, 2009]. The mechanisms for the adverse adrenergic effects as a result of chronic beta stimulation, may include a series of events which include 1) an increased heart rate and contractility increases oxygen demand ( $MVO_2$ ) and promotes ischaemia; 2) Increased arterial blood pressure which is associated with an increased cardiac output which increases alpha-adrenergic mediated peripheral vascular resistance which exaggerates ischaemia and damages the vascular endothelium and 3) The increased circulation of free fatty acids have harmful effects on membranes and promote platelet aggregation. All these links to the onset of AMI and even if AMI is not triggered, there is an early increase in circulating catecholamine levels. Much evidence supports the concept that excessive beta-adrenergic activation can exaggerate the degree of myocardial ischaemia [Verrier et al., 1974; Lown et al., 1973; Corbalan et al., 1976]. An increase in heart rate increases the severity of ischaemic injury [Schaper, 1987; Verrier et al., 1974] and increases the incidence of reperfusion arrhythmias [Lederman, 1987]. Studies indicated that two agents known to reduce heart rate, namely propranolol and the calcium antagonist diltiazem, decreased reperfusion calcium uptake in the case of propranolol [Miyazawa 1986] and reperfusion arrhythmias [Tosaki & Hearse, 1987].

Energy metabolism is enhanced in animals during stressful events and is a characteristic of the “fight or flight” response and lead to deprivation of energy stores as seen in the IPC hearts (glycolysis is attenuated and glucose and fatty acid biosynthesis is enhanced). Since the animals in both groups experienced psychological stress and only the IPC group are energy deprived, it could be that the multiple short episodes of ischaemia of the IPC protocol, utilized or consume more energy via anaerobic glycolysis and depleted the energy stores of the IPC hearts compared to the Non-IPC hearts. As a result the IPC hearts had lower

energy stores at the start of sustained ischaemia to keep the ischaemic tissue viable via anaerobic glycolosis during the 25 min of sustained ischaemia.

These results indicate that animals in both groups experienced psychological stress before the hearts were excised, but the non-IPC could reverse the stress, as indicated by the unaltered metabolism and also by upregulating the protective heat shock proteins and anti-apoptotic proteins. The results suggest that psychological stress may cause the IPC hearts to be more susceptible to ischaemic damage during the period of sustained ischaemia and increased oxidative stress compared to non-IPC hearts. In addition, we could also identify the proteins responsible for the downregulation of the MAPK signaling pathway in the IPC hearts.

Taken together, these results demonstrated that an IPC heart which failed after ischaemia /reperfusion is metabolically compromised and “worse off” compared to a non-IPC heart. To our knowledge this is the only study that investigated this aspect in the context of IPC during reperfusion.

In addition, the results from this part of the study suggests that enhanced psychological stress and consequent increase in sympathetic stimulation (before repetitive short episodes of ischaemia) can result in poor recovery of the heart, after a prolonged period of sustained ischaemia.

## Chapter 5

### **An investigation of the variable signaling patterns of kinase activation before and after IPC and in control hearts before and after an ischaemic insult.**

#### **5.1) Introduction**

Studies addressing the signal transduction pathways involved in cardioprotection by ischaemic preconditioning have focused increasingly on the role of the prosurvival kinases, including the PI3-kinase pathway and the extracellular signal-regulated kinases (ERK1/2). This is not surprising, since Hausenloy and co-workers (2005) have demonstrated that the cardioprotective effects of ischaemic preconditioning is completely abolished by inhibition of the RISK pathway. Furthermore, there is also increasing evidence that the cardioprotection afforded by preconditioning occurs during the early reperfusion phase after index ischaemia [Hausenloy et al., 2007].

The golden standard of the effectiveness of preconditioning is the reduction of the infarct size. However, there is also a series of other benefits obtained from preconditioning and among these is better recovery of post-ischaemic left ventricular function [Shiki & Hearse, 1987; Przyklenk, 2000; Yellon & Downey, 2003].

According to the literature, functional recovery of the ischaemic heart in IPC is associated with an attenuation of p38 activation after 30 min reperfusion [Marais et al., 2001]. However, our results did not follow the expected pattern of p38 MAPK activation, but showed variable responses between different animals. PKB/AKT also showed variable responses of activation between different animals, but to a lesser extent as p38 MAPK. However, in some groups of animals activation of the RISK pathway followed the same trend as activation of p38 MAPK.

This may suggest that:

(1) an attenuation of p38 MAPK kinase activation in IPC during reperfusion is detrimental to the ischaemic heart. The results in Chapter 3 of the thesis demonstrated that an attenuation of p38 MAPK in the IPC group after 15 min reperfusion is associated with an attenuation of both the protective PKB and HSP-27. This was accompanied by an attenuation in glucose metabolism and an upregulation of fatty acid metabolism, as demonstrated in Chapter 4 of the thesis.

(2) activation of the protective RISK pathway in the IPC group during 30 min reperfusion is not associated with functional recovery. The results in Chapter 3 of the thesis demonstrated that the RISK pathway was activated in the non-IPC hearts at 30 min reperfusion and was therefore associated with failing hearts.

In view of the above and to confirm the results obtained in Chapters 3 and 4, the aims of this part of the study were:

1) to correlate kinase activation with functional recovery in the IPC groups at 30 min reperfusion,

2) to investigate and identify the factors responsible for (or relating to) the variable responses of kinase activation observed in: (a) the IPC groups after reperfusion and directly after the IPC protocol before sustained ischaemia (b) Control hearts before and after sustained ischaemia.

## 5.2 Methods (Figure 5.1)

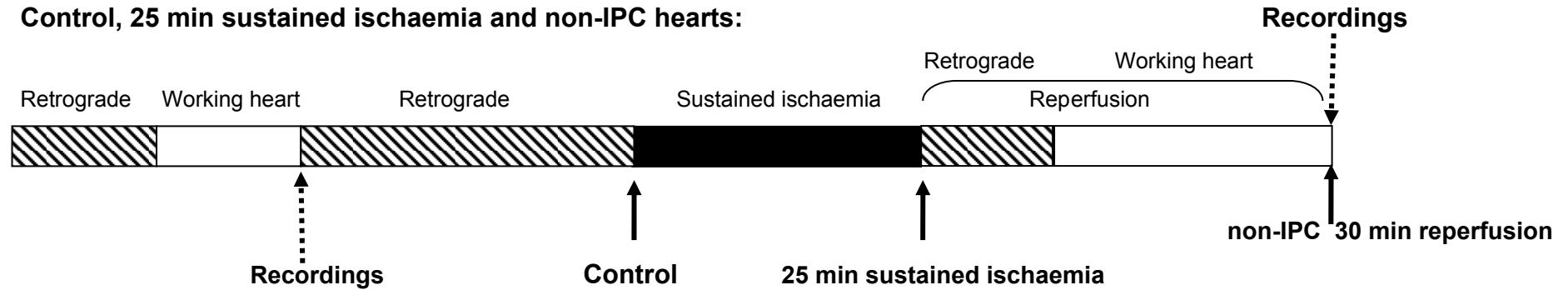
(i) **Control:** Hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde). The haemodynamic parameters of the hearts were recorded after 15 min working heart as indicated by the dashed arrows, as indicated in Fig. 5.1. Hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows (Fig.5.1).

ii) **25 min sustained ischaemia:** Hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde), followed by 25 min global sustained ischaemia. The haemodynamic parameters of the hearts were recorded after 15 min working heart, as indicated by the dashed arrows (Fig. 5.1). Hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows (Fig.5.1).

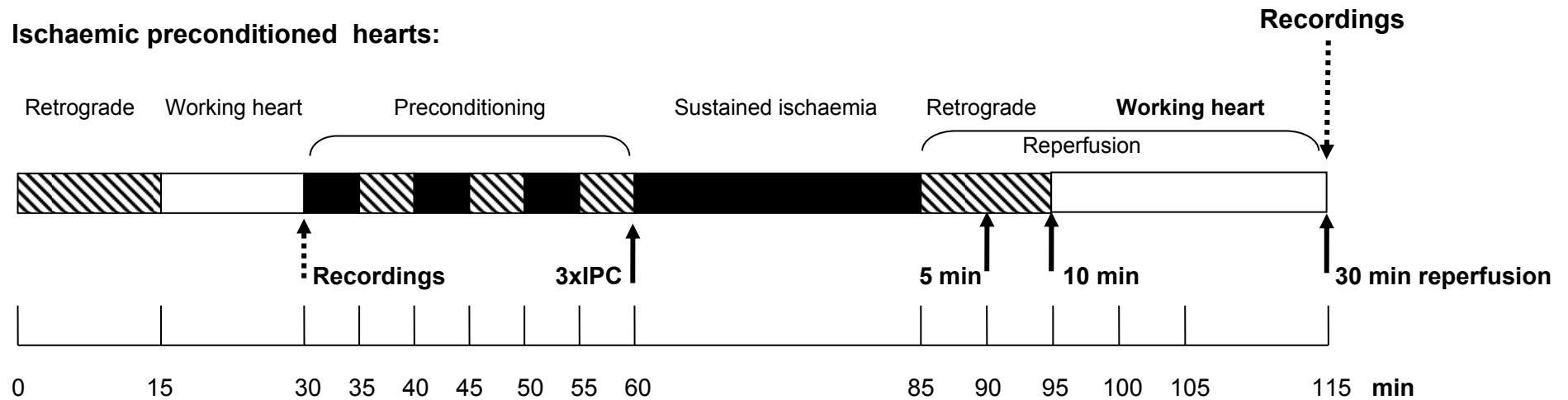
iii) **Ischaemic preconditioning (IPC):** Hearts were stabilized for 30 min (15 min retrograde, 15 min working heart) and then preconditioned by 3x5 min global ischaemia, alternated with 5 min reperfusion. Hearts were subjected to 25 min sustained global ischaemia, followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, the haemodynamic parameters of the hearts were recorded (indicated by the dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows in Fig.5.1. The haemodynamic parameters of the hearts were recorded after 15 min working heart, as indicated by the dashed arrows (Fig. 5.1). In order to investigate shorter reperfusion periods, hearts were snap-frozen at 5 and 10 min reperfusion, as indicated by the solid arrows in Fig. 5.1. In order to study events during the IPC period in the absence of sustained ischaemia and reperfusion, hearts were snap-frozen directly after the IPC period, indicated as 3xIPC (solid arrows in Fig.5.1).

iv) **Non-preconditioning (non-IPC):** Hearts were stabilized for 60 min (15 min retrograde, 15 min working heart and 30 min retrograde perfusion). Haemodynamic recordings were made at 15 min working heart as indicated by the dashed arrows in Fig. 5.1. Hearts were subsequently subjected to 25 min sustained global ischaemia followed by 30 min reperfusion (10 min retrograde perfusion and 20 min working heart). After 30 min reperfusion, haemodynamic recordings were made (dashed arrows) and hearts were snap-frozen for Western blotting analysis, as indicated by the solid arrows in Fig.5.1.

**Control, 25 min sustained ischaemia and non-IPC hearts:**



**Ischaemic preconditioned hearts:**



**Figure 5.1**

Experimental protocol according to a timeframe (min) indicating at which time intervals (indicated by solid arrows,  $\uparrow$ ), hearts were snap-frozen for western blot analysis. Functional recordings were made after 15 min working heart and 30 min reperfusion as indicated by the dashed arrows ( $\uparrow$ ).



## **5.3) Results**

### **5.3.1 The variable patterns of p38 MAPK activation in three IPC groups after 30 min reperfusion (Figures 5.3.1 A, B and C).**

Results from the present study did not follow the expected pattern of MAPK activation after 30 min reperfusion, but showed variable responses between different animals. All of these hearts (n=23 animals), from three different IPC groups showed functional recovery after the ischaemic insult.

### **5.3.2 The variable patterns of p38 MAPK, ERK and PKB/Akt activation in a fourth IPC group after 30 min reperfusion (Figures 5.3.2 A, B and C).**

Results from the present study, showed variable patterns of p38 MAPK activation after 30 min reperfusion, as indicated in **Fig. 5.3.2 A**. All of these hearts showed functional recovery after the ischaemic insult (n=8 animals). The percentage (%) functional recovery is indicated for each heart. The corresponding activation of ERK MAPK and PKB/Akt (denoted as the RISK pathway) is indicated in **Fig. 5.3.2 B** and **Fig. 5.3.2 C** respectively.

### **5.3.3 The variable patterns of p38 MAPK, ERK and PKB/Akt activation in a fifth IPC group after 30 min reperfusion (Figures 5.3.3 A, B and C).**

The results demonstrated the variable patterns of p38 MAPK activation after 30 min reperfusion in a different set of animals, indicated in **Fig. 5.3.3 A**. All of these hearts showed functional recovery after the ischaemic insult (n=8 animals). The percentage (%) functional recovery is indicated for each heart. The corresponding activation of ERK MAPK and PKB/Akt (denoted as the RISK pathway) is indicated in **Fig. 5.3.3 B** and **Fig. 5.3.3 C** respectively.

#### **5.3.4 The variable patterns of p38 MAPK, ERK and PKB/Akt activation in an IPC group that showed functional recovery and failed respectively, after 30 min reperfusion (Figures 5.3.4 A, B and C).**

The results demonstrated the variable activation patterns of p38 MAPK activation in the IPC group after 30 min reperfusion, indicated in **Fig. 5.3.4 A**. The percentage (%) functional recovery is indicated for each heart. Some hearts did not recover (failed) after the ischaemic insult, indicated by 0% (zero percent recovery). The corresponding activation patterns of ERK MAPK and PKB/Akt is indicated in **B** and **C** respectively. Interestingly, hearts that showed 49% and 50% functional recovery, showed in some animals the same level of p38 MAPK activation as failed hearts (**Figure 5.3.4 A**). Furthermore, PKB/AKT activation was more pronounced in failed hearts from some animals than in hearts that showed functional recovery (**Figure 5.3.4 C**).

#### **5.3.5 The variable patterns of p38 MAPK, ERK and PKB/Akt activation in an IPC group that failed after 30 min reperfusion (Figures 5.3.5 A, B and C).**

The results demonstrated the variable patterns of p38 MAPK activation in the IPC group after 30 min reperfusion, indicated in **Fig. 5.3.5 A**. These IPC hearts did not precondition and failed after 30 min reperfusion (indicated by 0 %). Interestingly hearts from this group of animals (n=8 animals) also showed a variable pattern of p38 MAPK activation (**Fig. 5.3.5 A**). The RISK pathway (**Fig. 5.3.5. B**) and (**Fig. 5.3.5 C**) was also activated in this group.

#### **5.3.6 The variable patterns of p38 MAPK, ERK and PKB/Akt activation in a non-IPC group after 30 min reperfusion (Figures 5.3.6 A, B and C).**

The results indicated the variable patterns of p38 MAPK activation in the non-IPC hearts after 30 min reperfusion, indicated in **Figure 5.3.6 A**. These hearts (n=8 animals) did not show functional recovery after 30 min reperfusion (indicated by 0%). Even more intriguing was the finding that the RISK pathway was also activated in the non-IPC hearts after 30 min reperfusion (**Figures 5.3.6 B and C**). In addition the small heat shock protein-27 (HSP-27) was also activated in the non-IPC group.

### 5.3.7 Activation patterns of p38 MAPK and the RISK pathway between an IPC group and non-IPC group at 30 min reperfusion (Figures 5.3.7 A, B, C and D).

The results in **Figure 5.3.7 A** demonstrated that at 30 min reperfusion p38 MAPK was significantly attenuated in IPC compared to non-IPC hearts ( $1.015 \pm 0.5167$  vs  $2.662 \pm 0.1096$ ;  $p < 0.05$ ). Activation of PKB/Akt was significantly lower in IPC compared to non-IPC ( $1.501 \pm 0.07889$  vs  $3.550 \pm 0.2426$ ;  $p < 0.05$ ), indicated in **Figure 5.3.7 B**. ERK p44 was significantly decreased in IPC compared to non-IPC ( $1.344 \pm 0.07658$  vs  $3.063 \pm 0.1312$ ;  $p < 0.05$ ). Similarly ERK p42 was also significantly attenuated in IPC compared to non-IPC ( $1.896 \pm 0.1168$  vs  $3.958 \pm 0.1678$ ;  $p < 0.05$ ), indicated in **Figure 5.3.7 C**. HSP-27 was also significantly attenuated in IPC compared to non-IPC ( $1.166 \pm 0.02704$  vs  $2.810 \pm 0.2270$ ;  $p < 0.05$ ) indicated in **Figure 5.3.7 D**.

### 5.3.8 Functional recovery and the RISK pathway (Figures 5.3.8 A, B and C)

The results from the present study indicated (**Figure 5.3.8a**) that no correlation ( $r = -0.376$ ) exists between activation of PKB/Akt and functional recovery in the IPC group after 30 min reperfusion. The results in **Figure 5.3.8b** indicated that there was no correlation ( $r = 0.0738$ ) between ERK p44 activation at 30 min reperfusion and functional recovery in the IPC group after 30 min reperfusion. Similarly no correlation ( $r = -0.07296$ ) was observed between activation of ERK p42 and functional recovery in the IPC group at 30 min reperfusion (**Figure 5.3.8c**).

### 5.3.9 Functional recovery and p38 MAPK (Figures 5.3.9)

Interestingly, there was also no correlation ( $r = -0.0637$ ) between activation of p38 MAPK and functional recovery in the IPC group at 30 min reperfusion (**Figure 5.3.9a**).

### 5.3.10 Correlation of the RISK pathway with haemodynamic parameters (Figures 5.3.10 a to 5.3.10 j)

Results from the present study indicated that activation of PKB/Akt in the IPC group at 5 min reperfusion correlated negatively ( $r = -0.8734$ ) with the aortic diastolic pressure at baseline/stabilisation (15 min working heart), **Figure 5.3.10a**. In addition, activation of PKB/Akt in the IPC group at 5 min reperfusion correlated positively ( $r = 0.7517$ ) with dP/dTmin measured at baseline/stabilisation (15 min working heart), **Figure 5.3.10b**. Activation of PKB/Akt did not correlate with any other haemodynamic parameters in the IPC group at this particular time point, but correlated positively ( $r = 0.7650$ ) in the IPC group at 10 min reperfusion with PSP measured at baseline/stabilisation (15 min working heart), **Figure 5.3.10c**. In addition, activation of PKB/Akt after the IPC protocol (3xIPC) correlated negatively ( $r = -0.7633$ ) with PSP measured at baseline/stabilisation (15 min working heart), (**Figure 5.3.10d**) and correlated positively ( $r = 0.7855$ ) with the Aortic diastolic pressure at baseline/stabilisation (15 min working heart), **Figure 5.3.e**. Interestingly, activation of PKB/Akt in the IPC group at 30 min reperfusion correlated positively ( $r = 0.578$ ) with heart rate at baseline/stabilisation (15 min working heart), **Figure 5.3.10f**.

Activation of ERK p42 after the IPC protocol (3xIPC) before sustained ischaemia, correlated negatively ( $r = -0.8131$ ) with aortic output (AO) measured at baseline/stabilisation (15 min working heart), **Figure 5.3.10g**. Activation of ERKp42 after the IPC protocol (3xIPC) before sustained ischaemia, correlated negatively ( $r = -0.7073$ ) with coronary output (CO) measured at baseline/stabilisation (15 min working heart), (**Figure 5.3.10h**). In addition, activation of ERK p42 in the IPC group at 30 min reperfusion correlated negatively ( $r = -0.64$ ) with total work measured at baseline/stabilisation (15 min working heart), **Figure 5.3.10i**. At the same timepoint (30 min reperfusion), activation of ERK p42 in the IPC group correlated positively ( $r = 0.58$ ) with heart rate measured at baseline/stabilisation (15 min working heart), **Figure 5.3.10j**.

### **5.3.11 Correlation between p38 MAPK, HSP and haemodynamic parameters (Figures 5.3.11a, b, c, d, & e)**

The results from the present study demonstrated that activation of p38 MAPK after 25 min ischaemia, correlated positively ( $r = 0.7089$ ) with aortic output (AO) measured at baseline/stabilisation (15 min working heart), **Figure 5.3.11a**. In addition, activation of p38 MAPK after the IPC protocol (3xIPC) before sustained ischaemia correlated negatively ( $r = -0.7265$ ) with total work measured at baseline/stabilisation (15 min reperfusion), **Figure 5.3.11b**.

Expression of HSP-70 after 30 min retrograde perfusion (control) correlated negatively ( $r = -0.7119$ ) with  $dP/dT_{Min}$  measured at baseline/stabilisation (15 min working heart), **Figure 5.3.11c**. Expression of HSP-70 after 30 min retrograde perfusion (control) correlated negatively ( $r = 0.7464$ ) with heart rate measured at baseline/stabilisation (15 min working heart), **Figure 5.3.11d**. Interestingly, expression of HSP-70 in the IPC group after 10 min reperfusion correlated positively ( $r = 0.7911$ ) with heart rate measured at baseline/stabilisation (15 min working heart), **Figure 5.3.11e**.

### **5.3.12 Correlation between CREB and haemodynamic parameters (Figure 5.3.12)**

The results from the present study demonstrated that activation of CREB after 25 min sustained ischaemia correlated positively ( $r = 0.7097$ ) with  $dP/dT_{Max}$  measured at baseline/stabilisation (15 min working heart), **Figure 5.3.12**.

### **5.3.13 Correlation between GSK-3 $\beta$ and haemodynamic parameters (Figure 5.3.13)**

Activation of GSK-3 $\beta$  in the IPC group after 30 min reperfusion correlated positively ( $r = 0.62$ ) with heart rate measured at baseline/stabilisation (15 min working heart), **Figure 5.3.13**.

### 5.3.14 Correlation between AMPK and haemodynamic parameters

(Figure 5.3.14 a,b & c)

Activation of AMPK after 30 min retrograde perfusion (control) correlated negatively ( $r = -0.8127$ ) with  $dP/dT_{Max}$  measured at baseline/stabilisation (15 min working heart), **Figure 5.3.14a**. Activation of AMPK after the IPC protocol (3xIPC) before sustained ischaemia correlated positively ( $r = 0.7488$ ) with aortic diastolic pressure measured at baseline/stabilisation (15 min working heart), **Figure 5.3.14b**. In addition, activation of AMPK after the IPC protocol (3xIPC) correlated negatively ( $r = -0.7753$ ) with peak systolic pressure measured at baseline/stabilisation (15 min working heart), **Figure 5.3.14c**.

### 5.3.15 Correlation between CAMKII and haemodynamic parameters

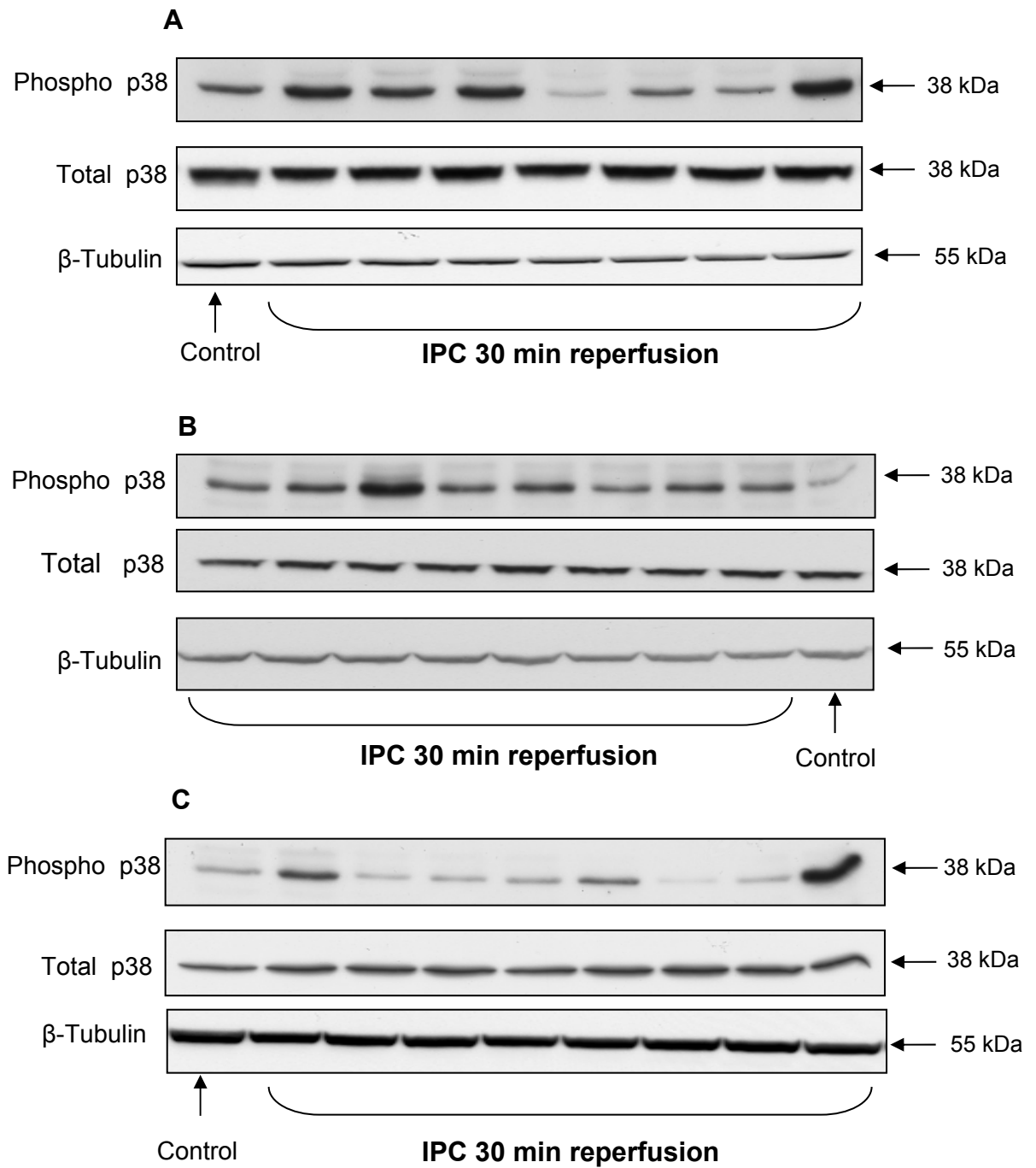
(Figure 5.3.15a, b & c)

Activation of CAMKII in the IPC group at 30 min reperfusion correlated negatively ( $r = -0.6303$ ) with aortic output (AO) measured at 30 min reperfusion (**Figure 5.3.15a**). Activation of CAMKII in the IPC group at 30 min reperfusion also correlated negatively ( $r = -0.6259$ ) with coronary output measured at 30 min reperfusion (**Figure 5.3.15b**). In addition, this activation of CAMKII in the IPC group correlated negatively ( $r = -0.6610$ ) with total work measured at 30 min reperfusion (**Figure 5.3.15c**).

### 5.3.16 Heart rate and ejection fraction at baseline/stabilisation (15 min working heart) in the IPC group after 30 min reperfusion (Figure 5.3.16 A & B)

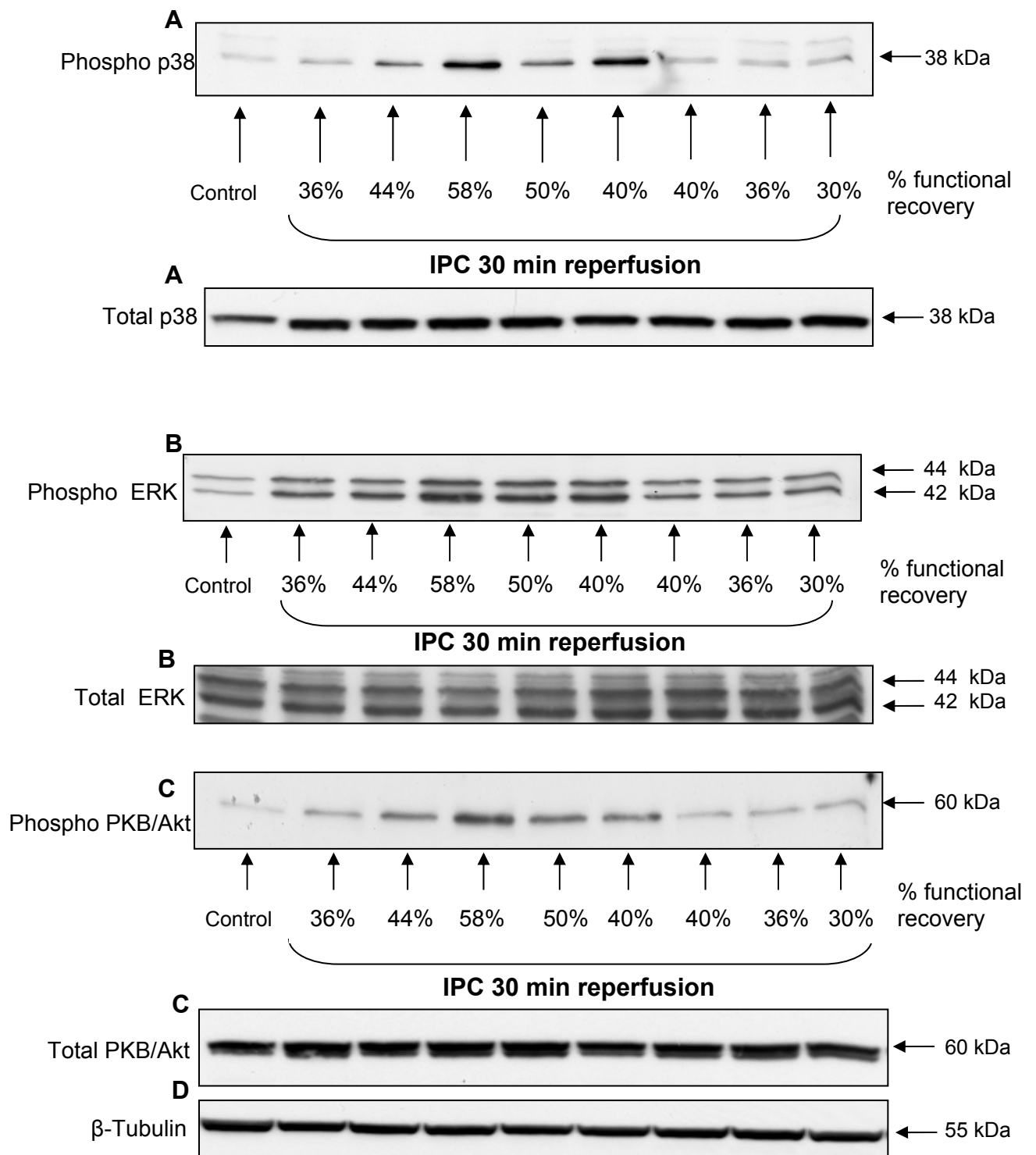
Results from the present study demonstrated that heart rate (HR) measured at baseline/stabilisation (15 min working heart) was significantly increased in the IPC group that failed after 30 min reperfusion, compared to the IPC group that showed functional recovery after 30 min reperfusion (**Figure 5.3.16A**). **Figure 5.3.16B** indicates the differences in ejection fraction between the same hearts as in **A**. Results demonstrated that the ejection fraction measured at baseline/stabilisation (15 min working heart) was significantly decreased in IPC

hearts that failed after 30 min reperfusion, compared to IPC hearts that showed functional recovery after 30 min reperfusion (**Figure 5.3.16B**).

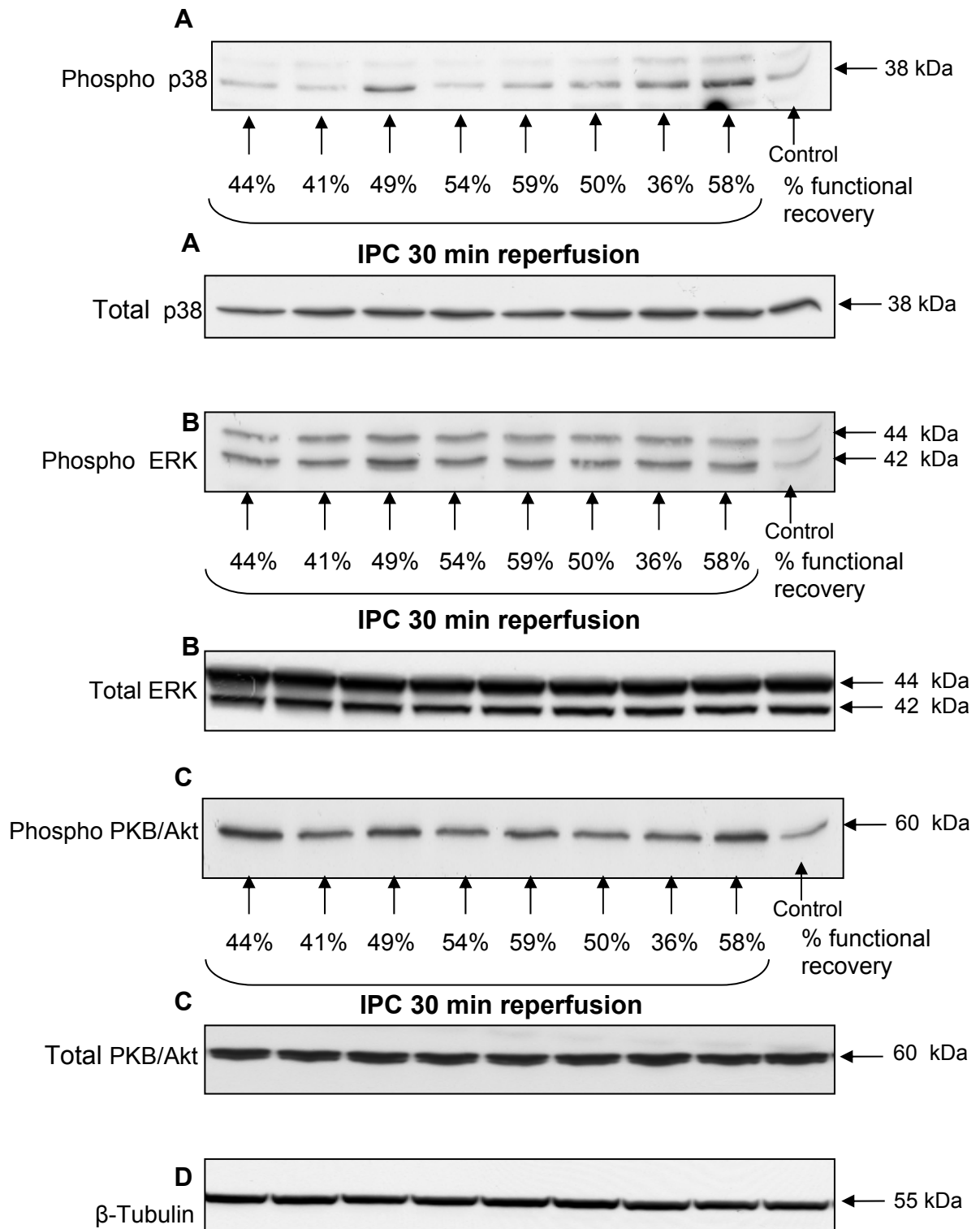


**Fig. 5.3.1. A, B and C. The variable patterns of p38 MAPK kinase activation in IPC hearts after 30 min reperfusion.** All of these hearts (n=23 animals) showed functional recovery after the ischaemic insult. The corresponding Total p38 and β-Tubulin blot is shown for each pospho-p38 blot.

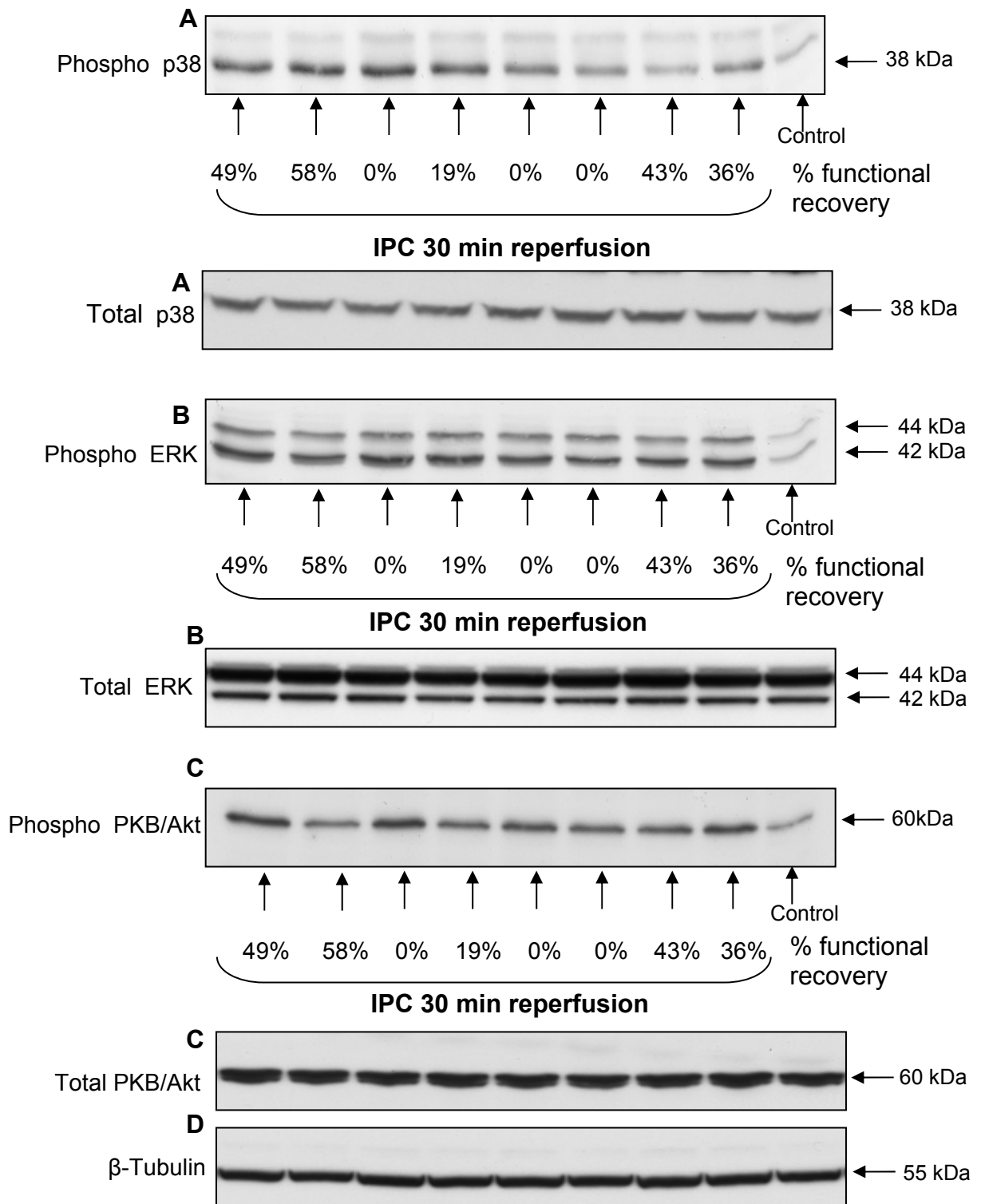




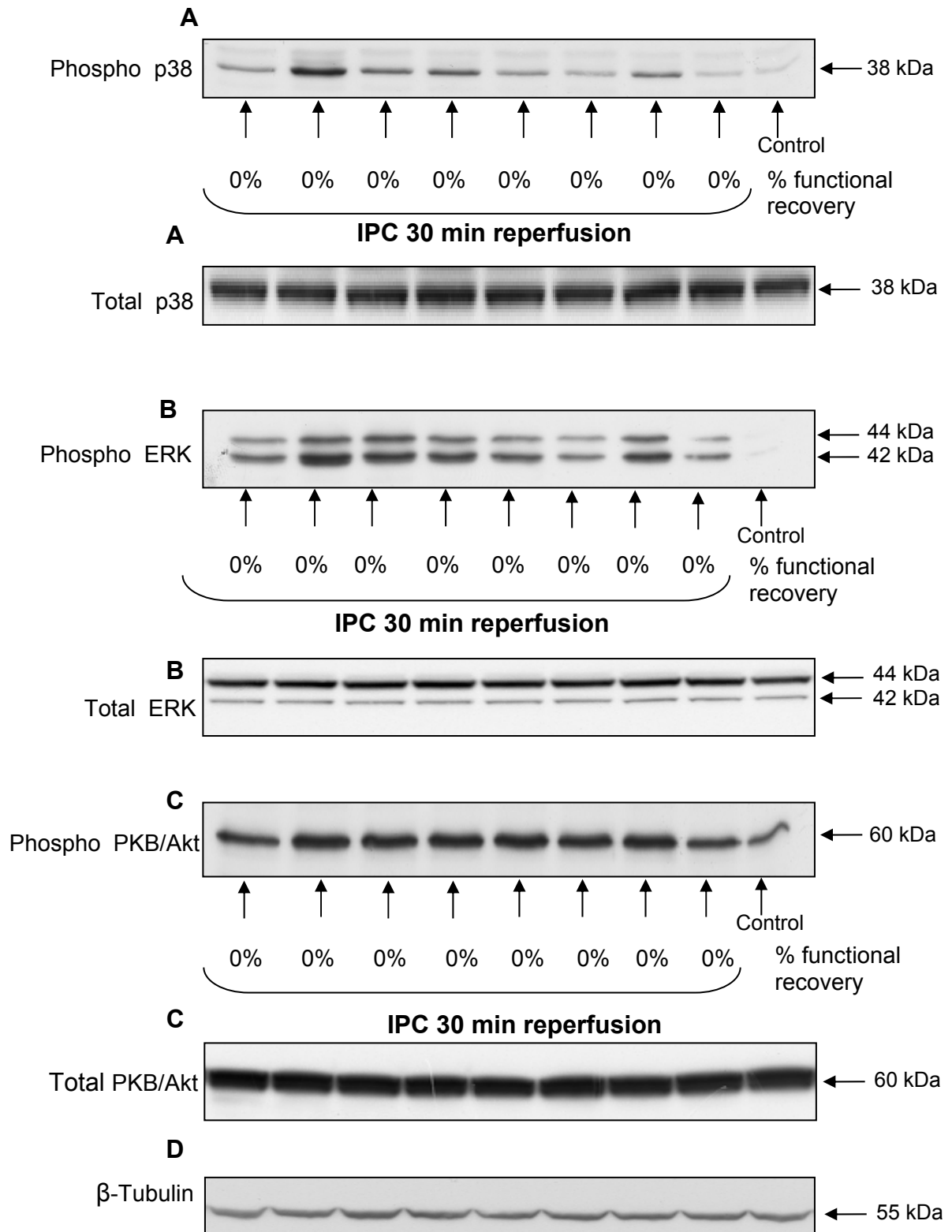
**Fig.5.3.2 The variable patterns of p38 MAPK activation in IPC hearts after 30 min reperfusion, indicated in A.** The percentage (%) functional recovery is indicated for each heart. All of these hearts (n=8 animals) showed functional recovery after the ischaemic insult. The corresponding activation of ERK MAPK and PKB/AKT is indicated in **B** and **C**, respectively.  $\beta$ -Tubulin is shown in **D**.



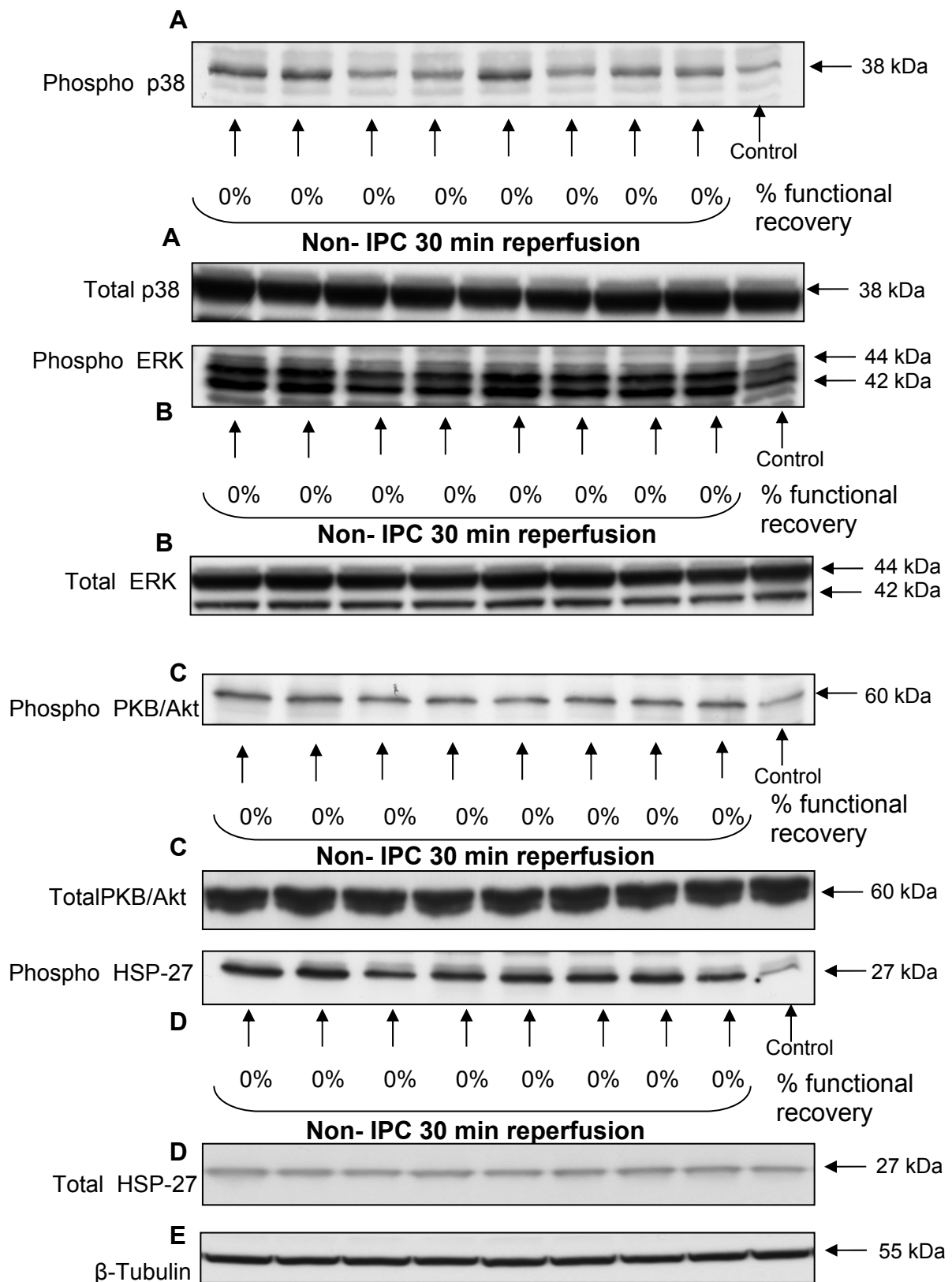
**Fig. 5.3.3 The variable patterns of p38 MAPK activation in IPC hearts after 30 min reperfusion, indicated in A.** The percentage (%) functional recovery is indicated for each heart. All of these hearts (n=8 animals) showed functional recovery after the ischaemic insult. The corresponding activation of ERK MAPK and PKB/AKT is indicated in **B** and **C**, respectively.  $\beta$ -Tubulin is shown in **D**.



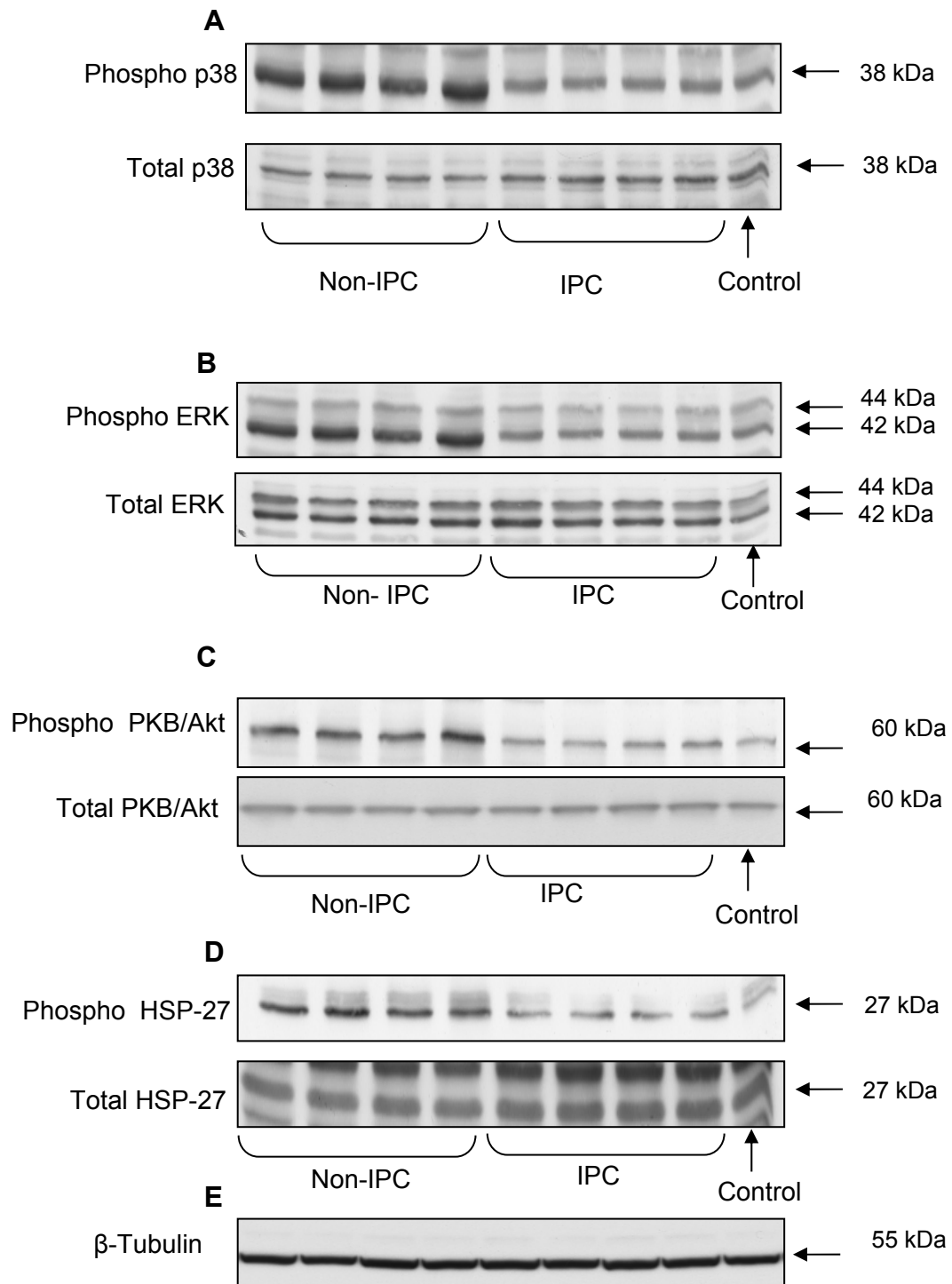
**Fig. 5.3.4 The variable patterns of p38 MAPK activation in IPC hearts after 30 min reperfusion, indicated in A.** The percentage (%) functional recovery is indicated for each heart. Some hearts did not recover (failed) after the ischaemic insult (indicated by 0%). The corresponding activation of ERK and PKB/Akt is indicated in **B** and **C**, respectively. β-Tubulin as loading control is shown in **D**.



**Fig. 5.3.5** The variable patterns of p38 MAPK activation in IPC hearts which failed (0% recovery) after 30 min reperfusion, indicated in **A**. The corresponding activation of ERK MAPK and PKB/Akt is shown in **B** and **C** respectively. β-Tubulin as loading control is shown in **D**.

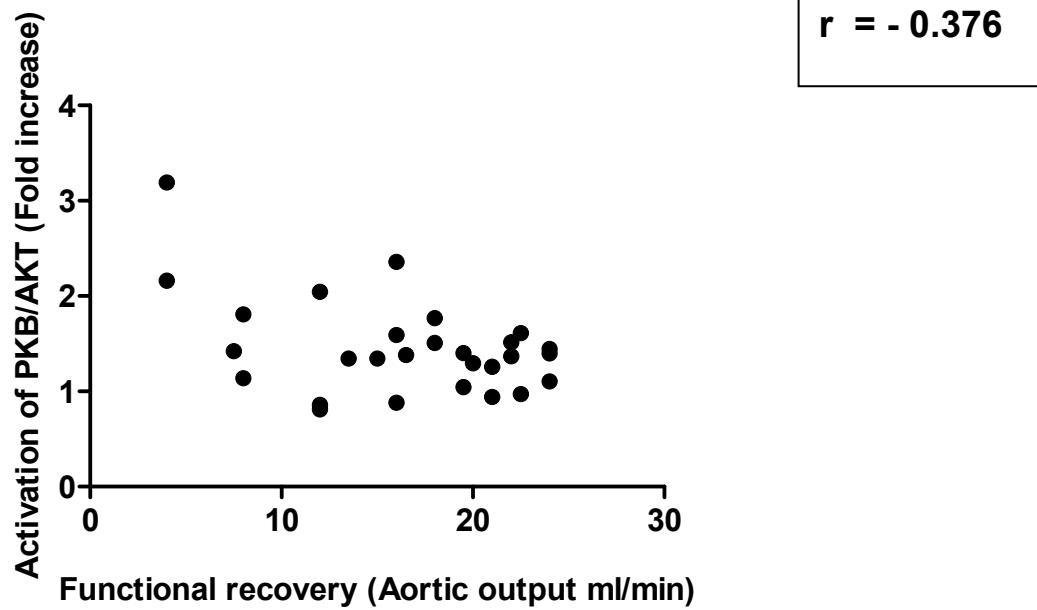


**Fig. 5.3.6 Variable patterns of p38 MAPK activation in non-IPC hearts after 30 min reperfusion, indicated in A.** The corresponding activation of ERK, PKB/Akt and HSP-27 is shown in B, C and D respectively.  $\beta$ -Tubulin as loading control is shown in E.

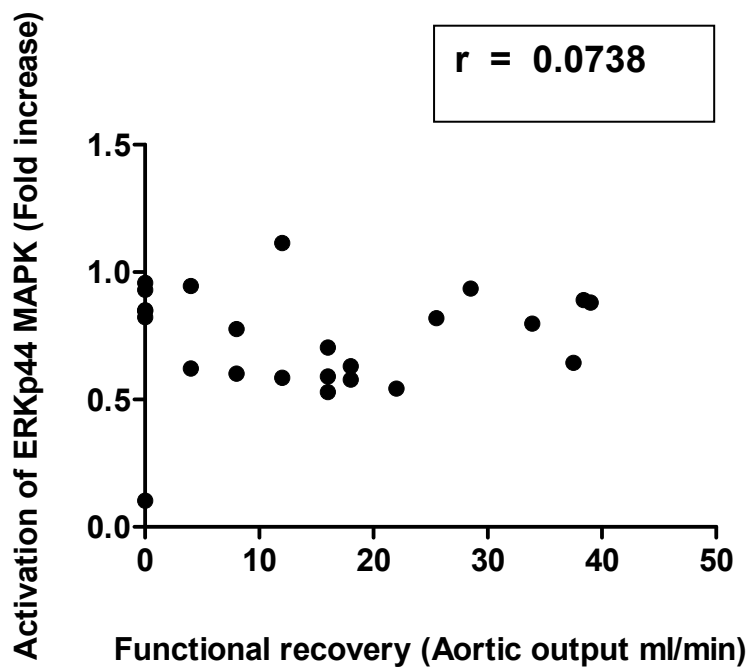


**Fig. 5.3.7. Activation of p38 (A), ERK (B), PKB/Akt (C) and HSP-27 (D) between non-IPC (n=4 animals) and IPC hearts (n=4 animals);  $p < 0.05$ .** IPC hearts showed functional recovery after 30 min reperfusion. β-Tubulin as loading control is shown in **E**.

### 5.3.8 Functional recovery and the RISK pathway



**Figure 5.3.8a** Correlation of PKB/AKT with functional recovery in the IPC group at 30 min reperfusion (n=28 animals).



**Figure 5.3.8b** Correlation of ERKp44 with functional recovery in the IPC group at 30 min reperfusion (n= 24 animals).

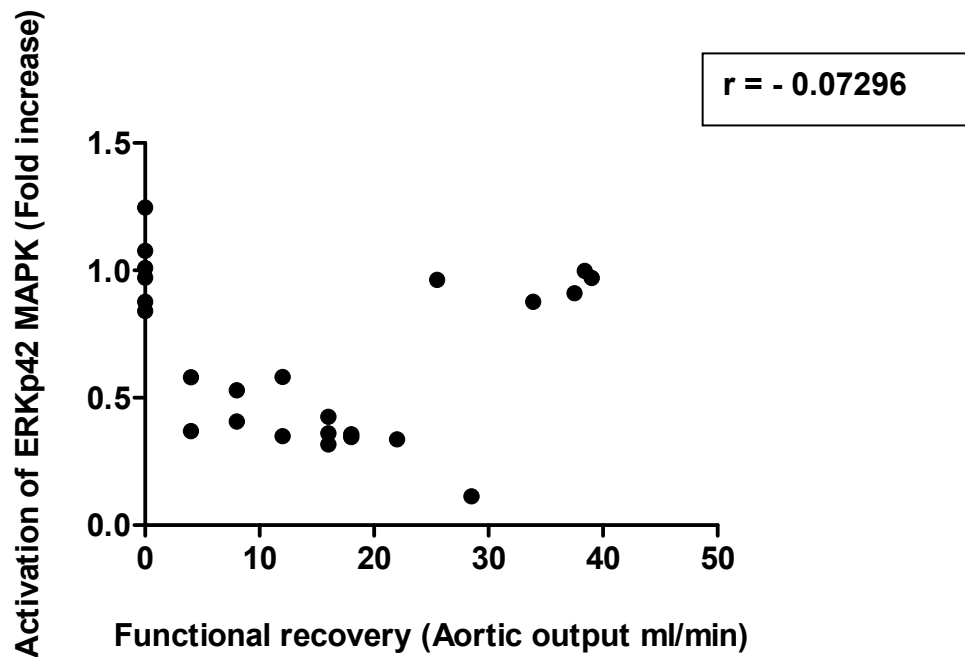


Figure 5.3.8c Correlation of ERKp42 with functional recovery in the IPC group at 30 min reperfusion (n = 24 animals).

### 5.3.9 Functional recovery and p38 MAPK

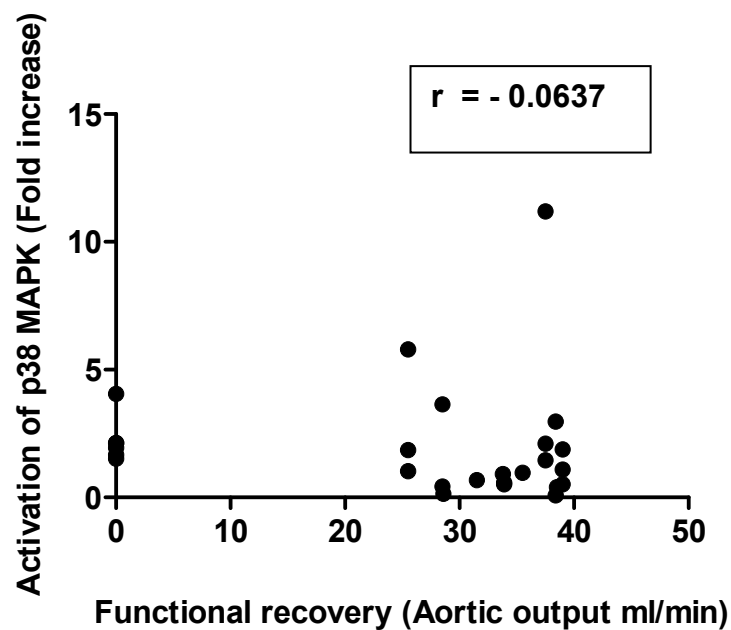


Figure 5.3.9a Correlation of p38 MAPK with functional recovery in the IPC group at 30 min reperfusion (n=26 animals).



### 5.3.10 Correlation of the RISK pathway with haemodynamic parameters

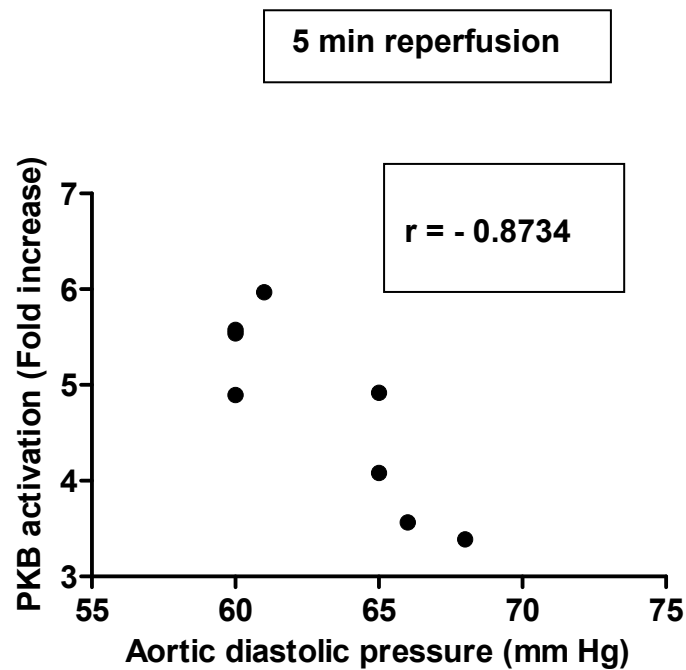


Figure 5.3.10a Correlation of PKB activation in the IPC group after 5 min reperfusion with Aortic diastolic pressure measured at baseline/stabilisation (15 min working heart), n=8.

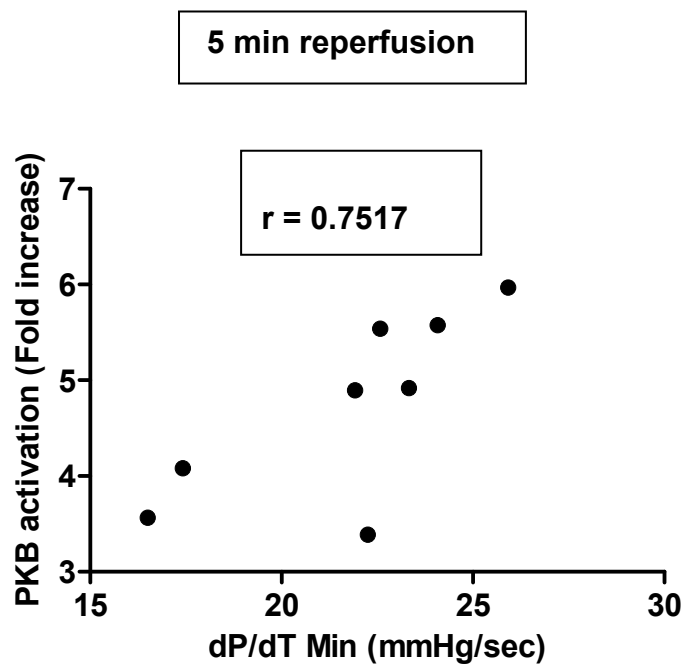


Figure 5.3.10b Correlation of PKB activation in the IPC group after 5 min reperfusion with dP/dT min measured at baseline/stabilisation (15 min working heart) (n=8).

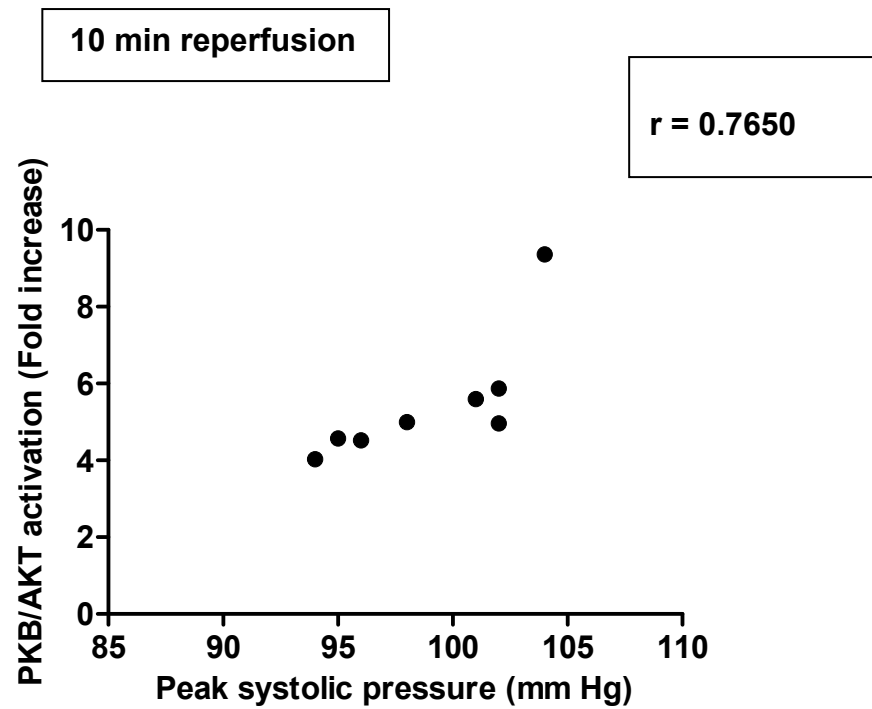


Figure 5.3.10c Correlation of PKB/Akt activation in the IPC group at 10 min reperfusion, with PSP measured at baseline/stabilisation (15 min working heart), n=8.

**After the IPC protocol, before sustained ischaemia**

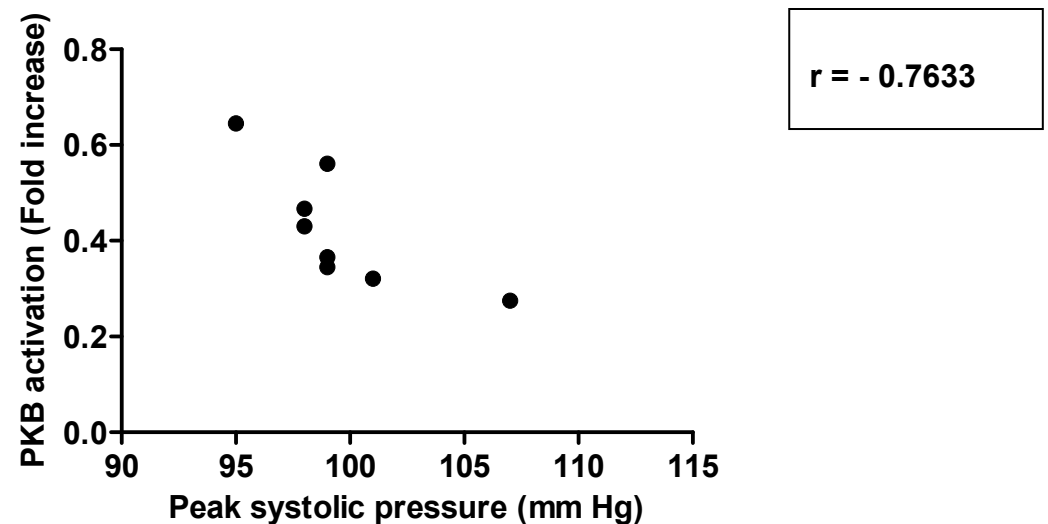


Figure 5.3.10d Correlation of PKB activation after 3x IPC with peak systolic pressure (PSP) measured at baseline/stabilisation (15 min working heart), n=8.

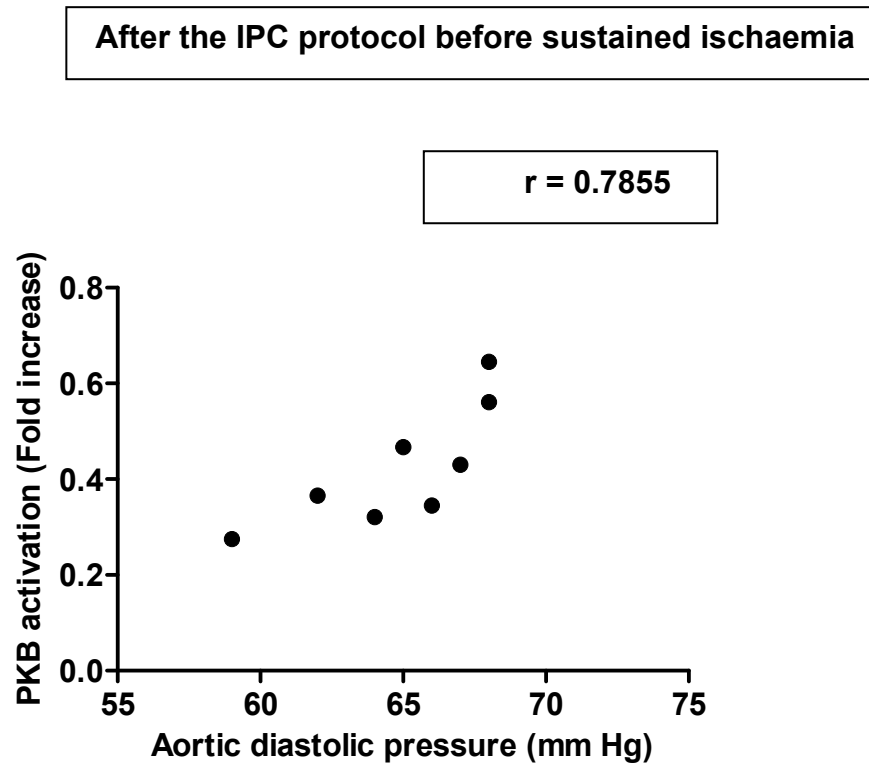


Fig. 5.3.10e Correlation of PKB activation during 3xIPC with Aortic diastolic pressure measured at baseline/stabilisation (15 min working heart), n=8.

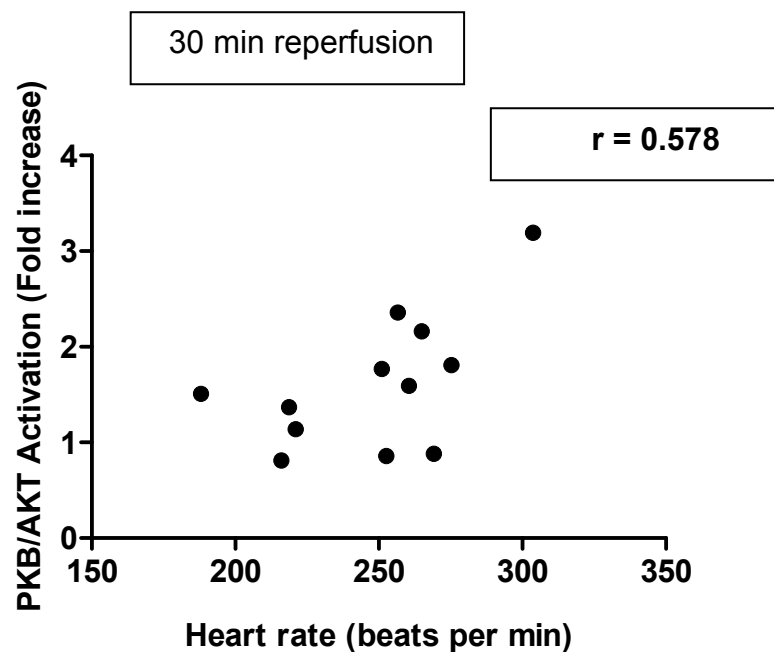


Fig. 5.3.10f Correlation of PKB activation in the IPC group at 30 min reperfusion with heart rate measured at baseline/stabilisation (15 min working heart), n=8.

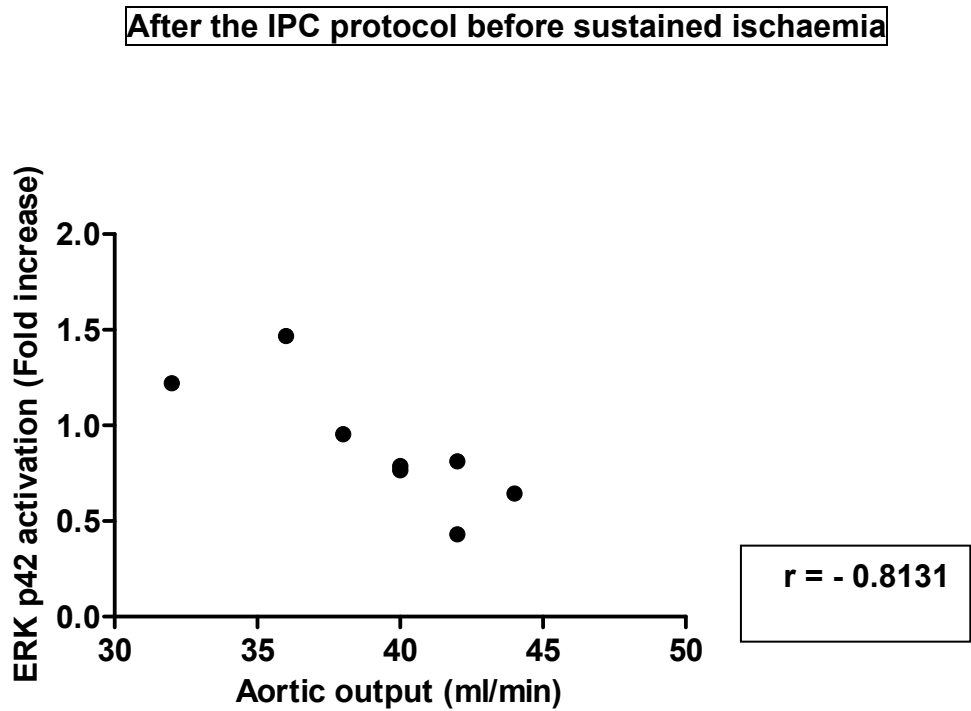


Figure 5.3.10g Correlation of ERK p42 activation after 3X IPC with Aortic output measured at baseline/stabilisation (15 min working heart), n=8.

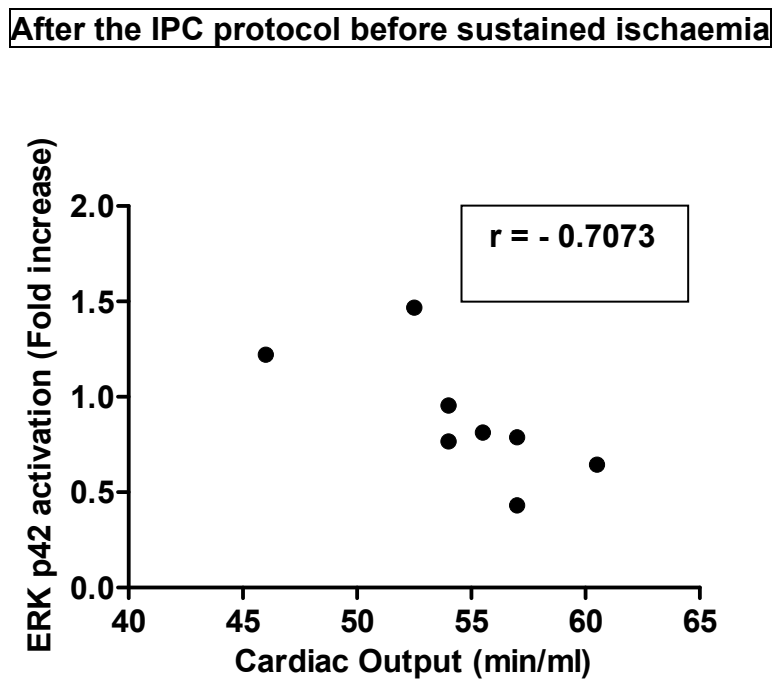


Figure 5.3.10h Correlation of ERK p42 activation after 3x IPC with cardiac output measured at baseline/stabilisation (15 min working heart), n=8.

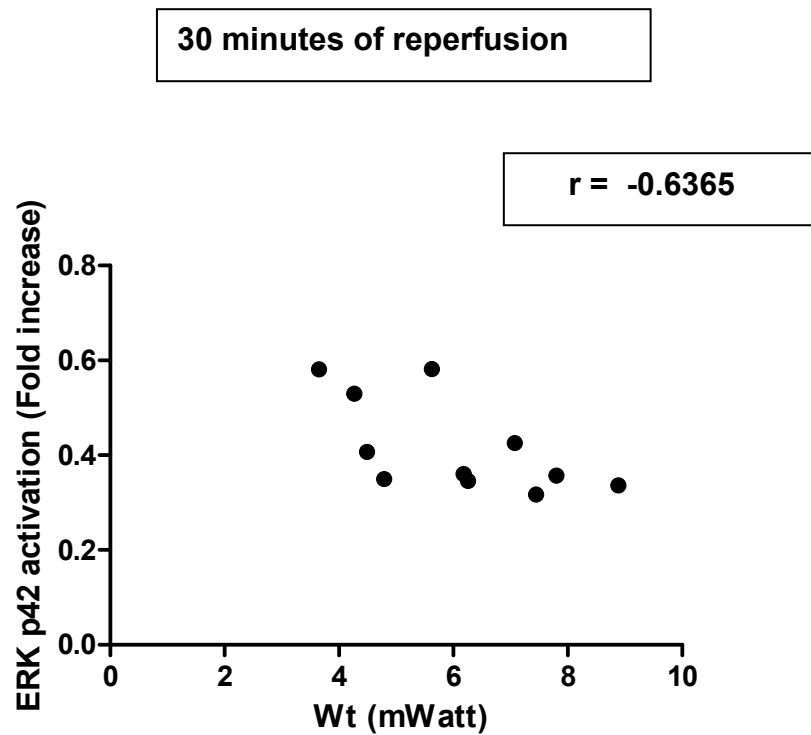


Figure 5.3.10i Correlation of ERK p42 activation in the IPC group at 30 min reperfusion with total work measured at baseline/stabilisation (15 min working heart), n=8.

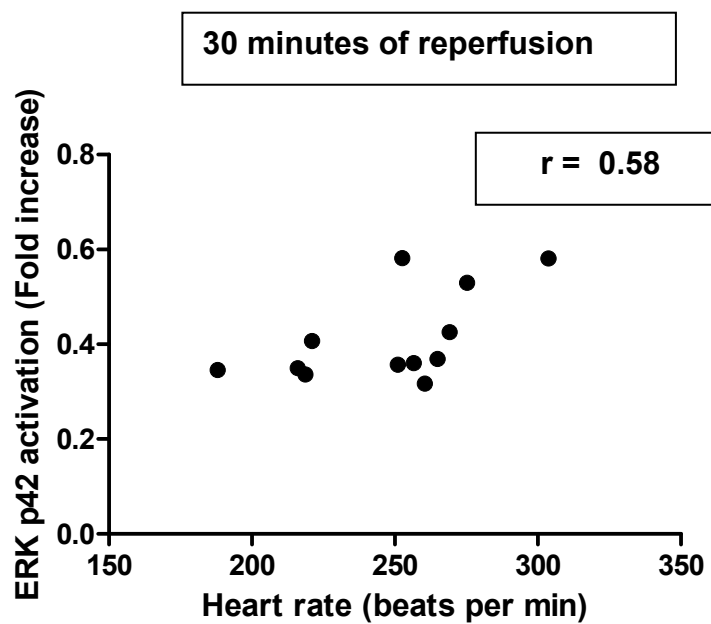


Figure 5.3.10j Correlation of ERK p42 activation in the IPC group at 30 min reperfusion with heart rate measured at baseline/stabilisation (15 min working heart), n=8.

### 5.3.11 Correlation between p38 MAPK, HSPs and haemodynamic parameters

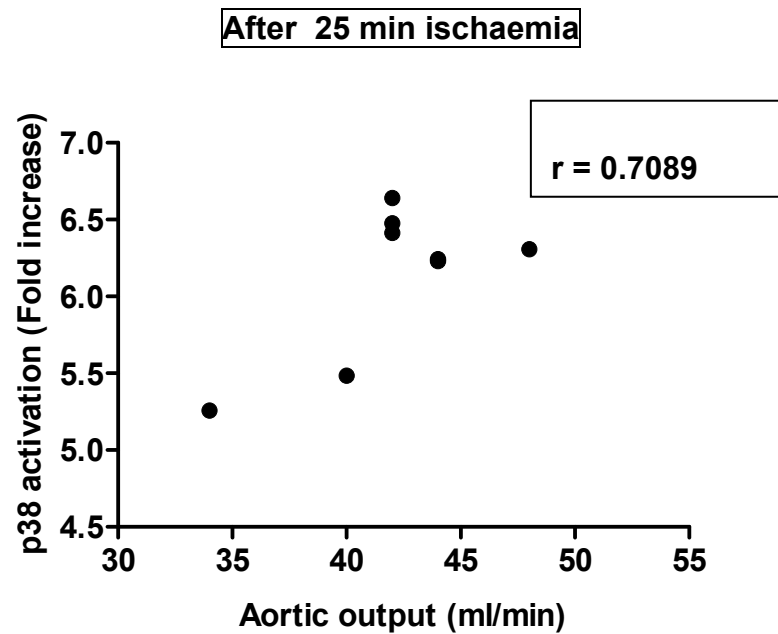


Figure 5.3.11a Correlation of p38 activation after 25 min global ischaemia with aortic output measured at baseline/stabilisation (15 min working heart), n=8.

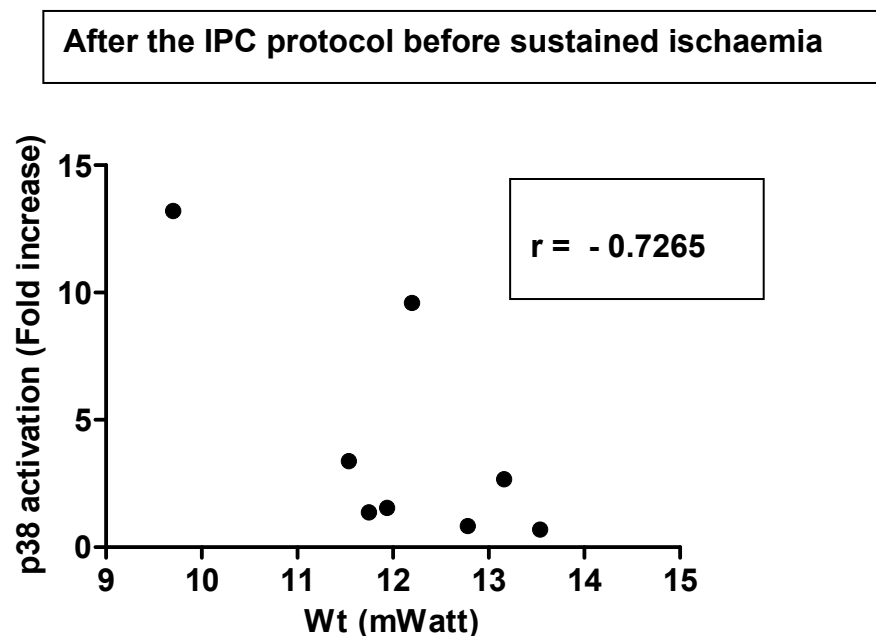


Figure 5.3.11b Correlation of p38 activation after 3xIPC with total work measured at baseline/stabilisation (15 min working heart), n=8.

**Control (30 min retrograde perfusion)**

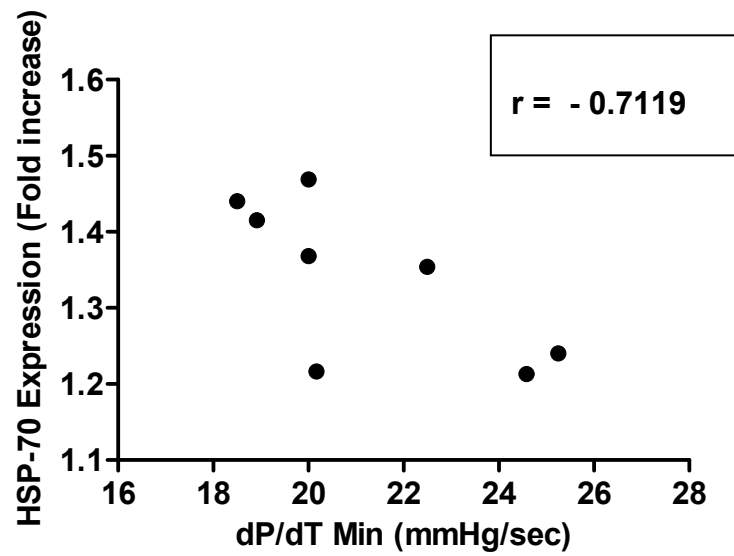


Figure 5.3.11c Correlation of HSP-70 expression after 30 min retrograde perfusion with dP/dT min measured at baseline/stabilisation (15 min working heart), n=8.

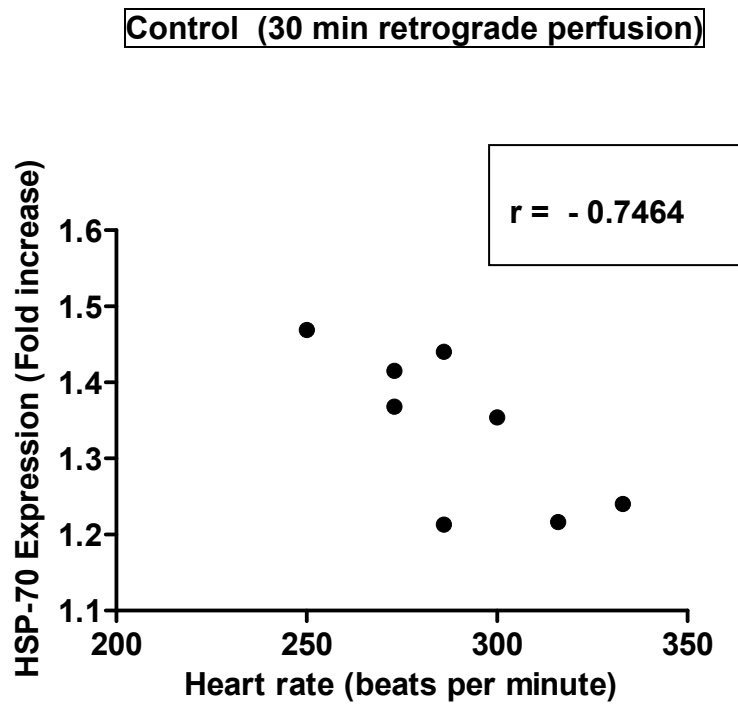


Figure 5.3.11d Correlation of HSP-70 expression after 30 min retrograde perfusion with heart rate measured at baseline/stabilisation (15 min working heart), n=8.

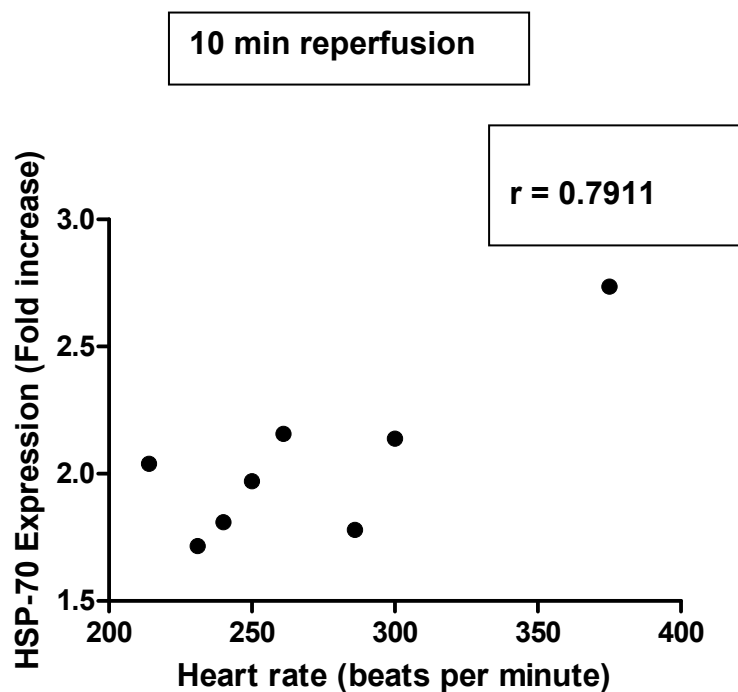


Figure 5.3.11e Correlation of HSP-70 expression in the IPC group at 10 min reperfusion with heart rate measured at baseline/stabilisation (15 min working heart), n=8.



### 5.3.12 Correlation between CREB and haemodynamic parameters

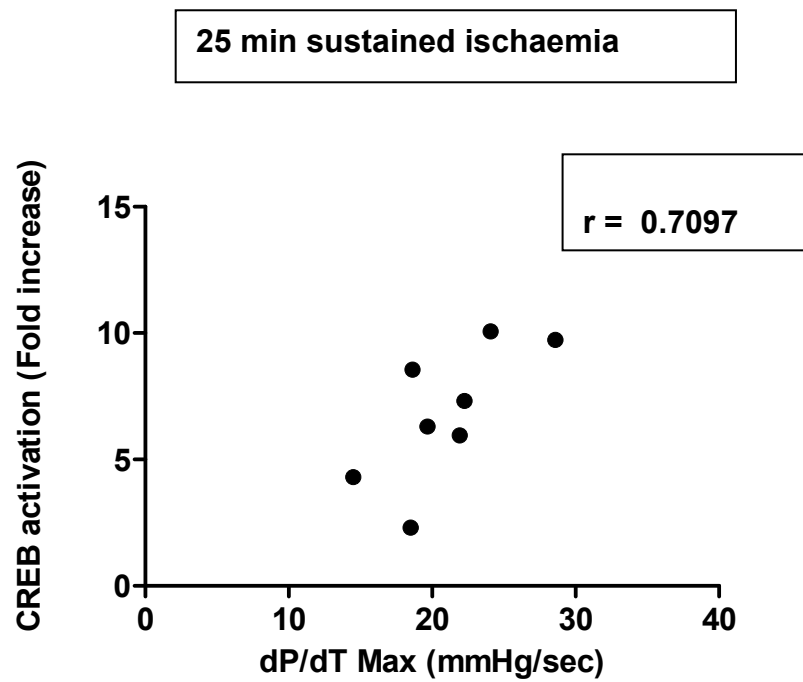


Figure 5.3.12 Correlation of CREB after 25 min sustained ischaemia with dP/dTMax measured at baseline/stabilisation (15 min working heart), n=8.

### 5.3.13 Correlation between GSK-3 $\beta$ and haemodynamic parameters

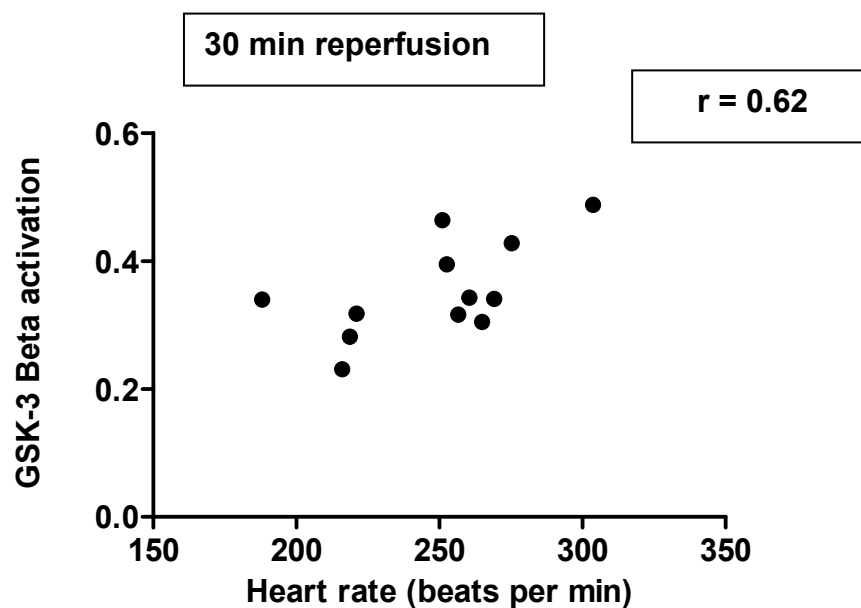


Figure 5.3.13 Correlation of GSK-3 $\beta$  in the IPC group at 30 min reperfusion with heart rate measured at baseline/stabilisation, 15 min working heart, n=8.

### 5.3.14 Correlation between AMPK and haemodynamic parameters

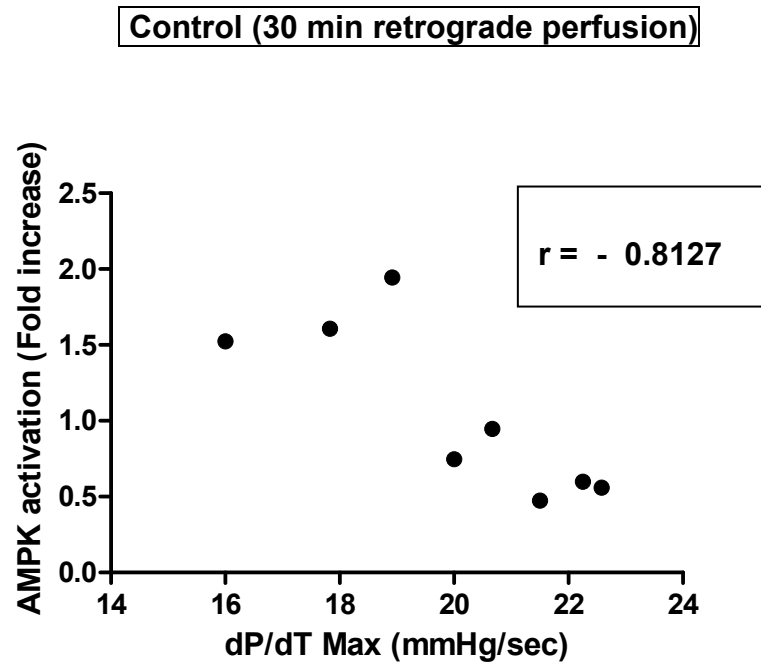


Figure 5.3.14a Correlation of AMPK after 30 min retrograde perfusion with dP/dTmax measured at baseline/stabilisation (15 min working heart), n=8.

**After the IPC protocol before sustained ischaemia**

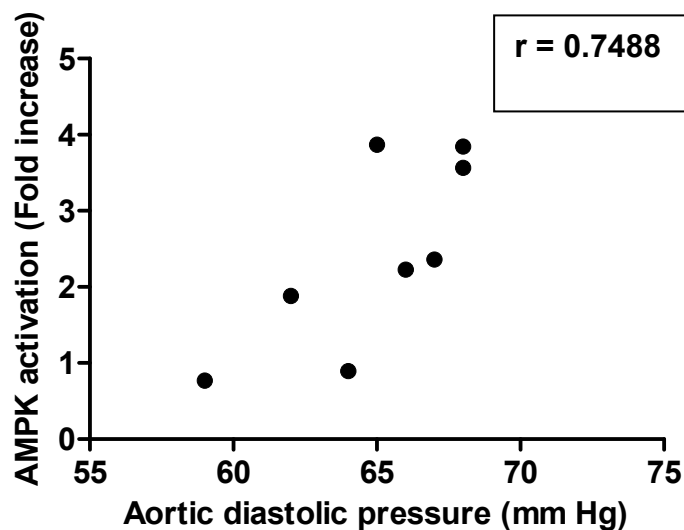


Figure 5.3.14b Correlation of AMPK after 3xIPC with Aortic diastolic pressure measured at baseline/stabilisation (15 min working heart), n=8.

### 5.3.14 Correlation between AMPK and haemodynamic parameters

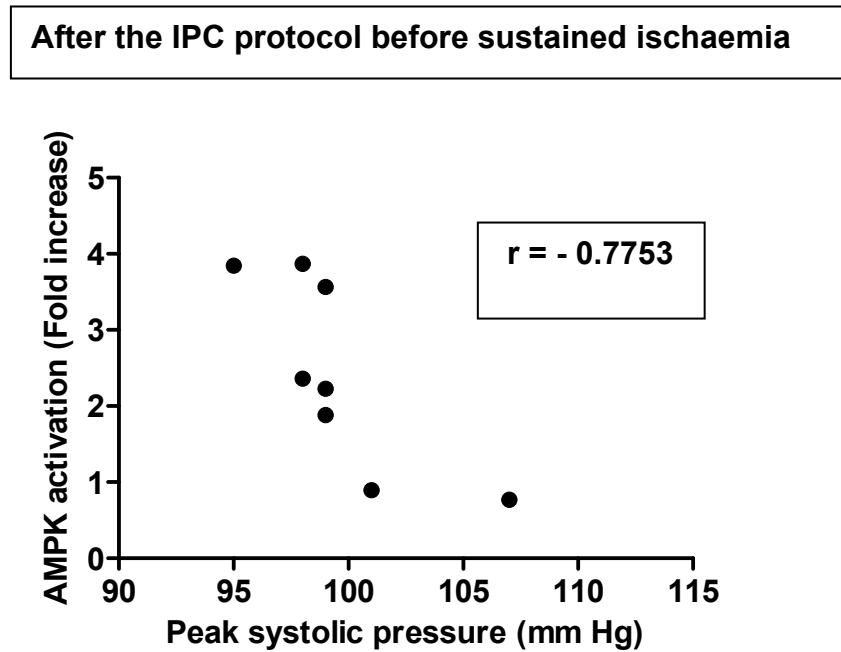


Figure 5.3.14c Correlation of AMPK after 3xIPC with PSP measured at baseline/stabilisation (15 min working heart), n=8.

### 5.3.15 Correlation between CAMKII and haemodynamic parameters

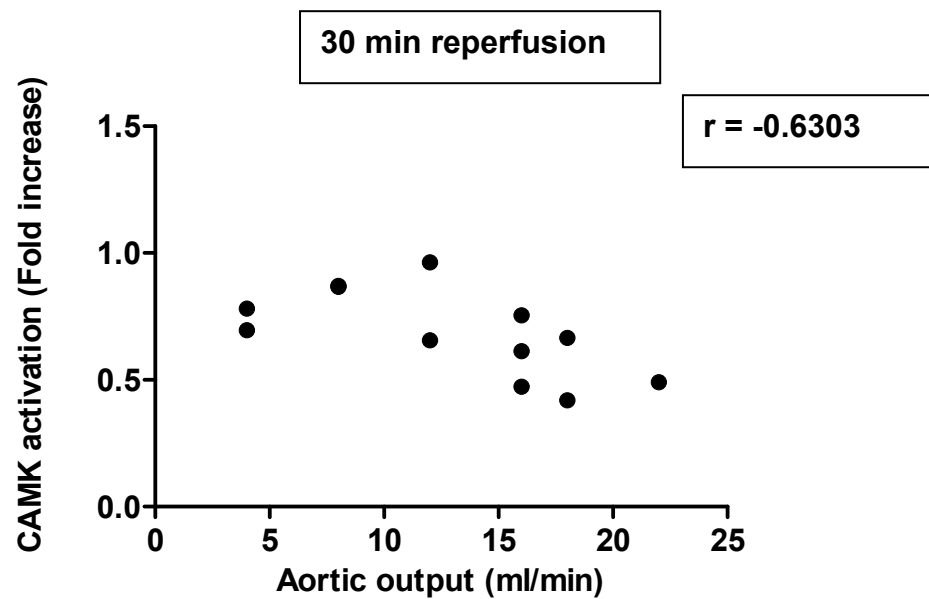


Figure 5.3.15a Correlation between CAMKII after 30 min reperfusion and aortic output at 30 min reperfusion (n=8).

### 5.3.15 Correlation between CAMKII and haemodynamic parameters

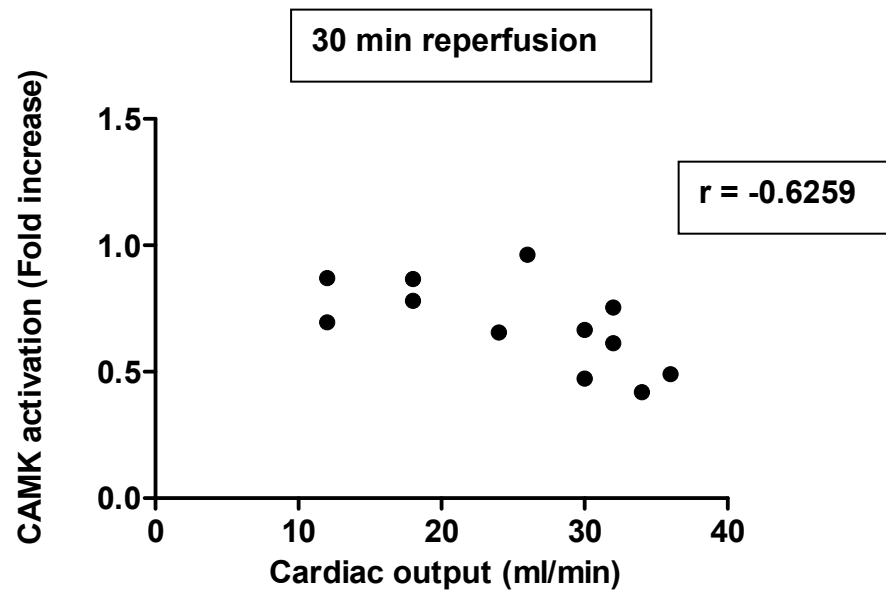


Figure 5.3.15b Correlation between CAMKII after 30 min reperfusion and cardiac output at 30 min reperfusion (n=8).

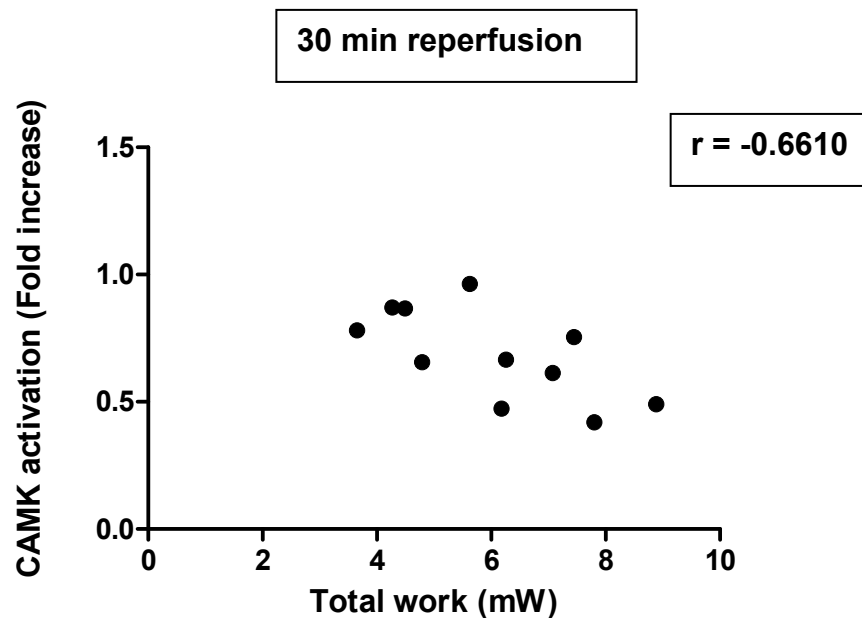


Figure 5.3.15c Correlation between CAMKII after 30 min reperfusion and total work at 30 min reperfusion (n=8).

**5.3.16 Comparison of the heart rate and ejection fraction obtained for the IPC group at baseline/stabilisation and functional recovery at 30 min reperfusion.**

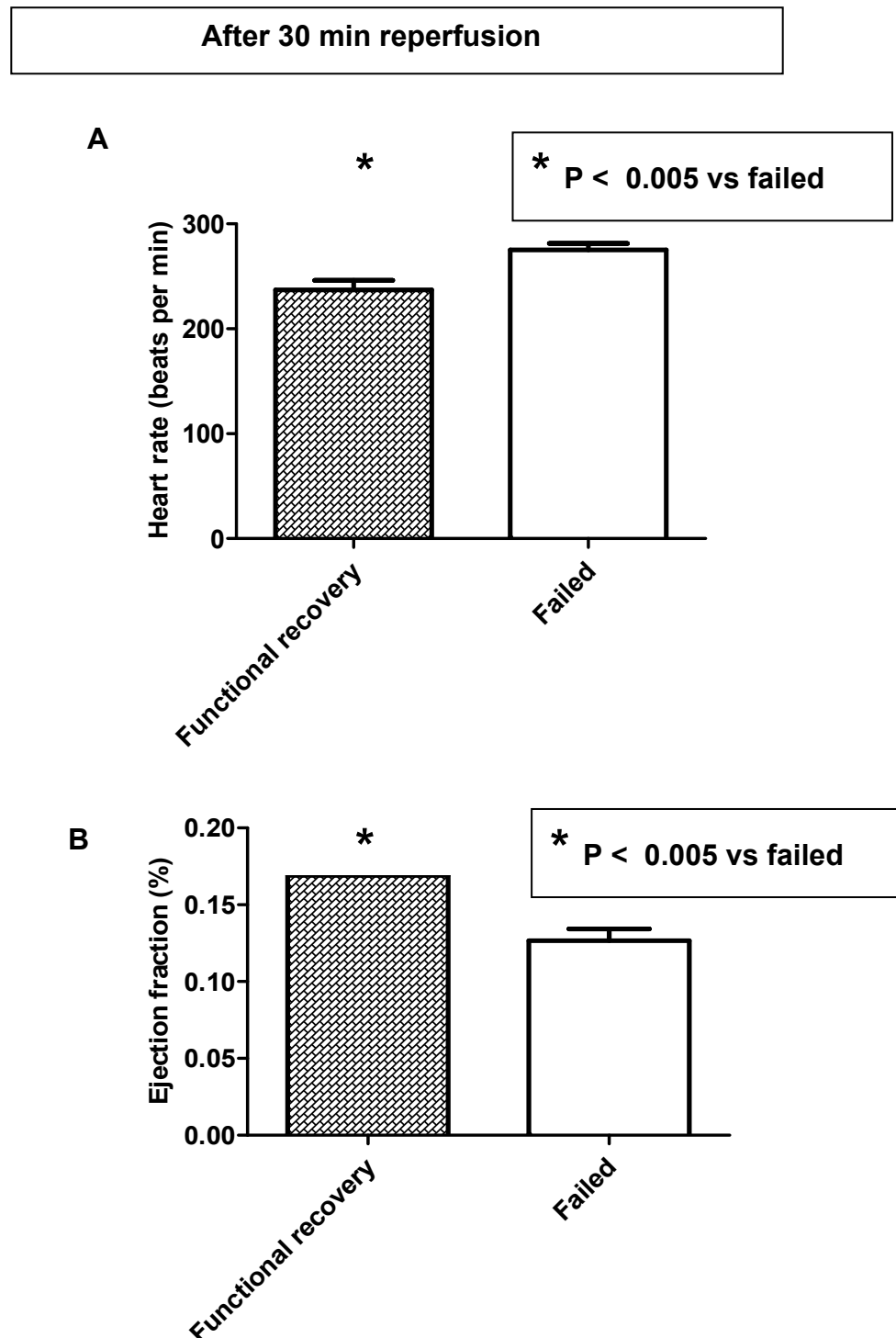
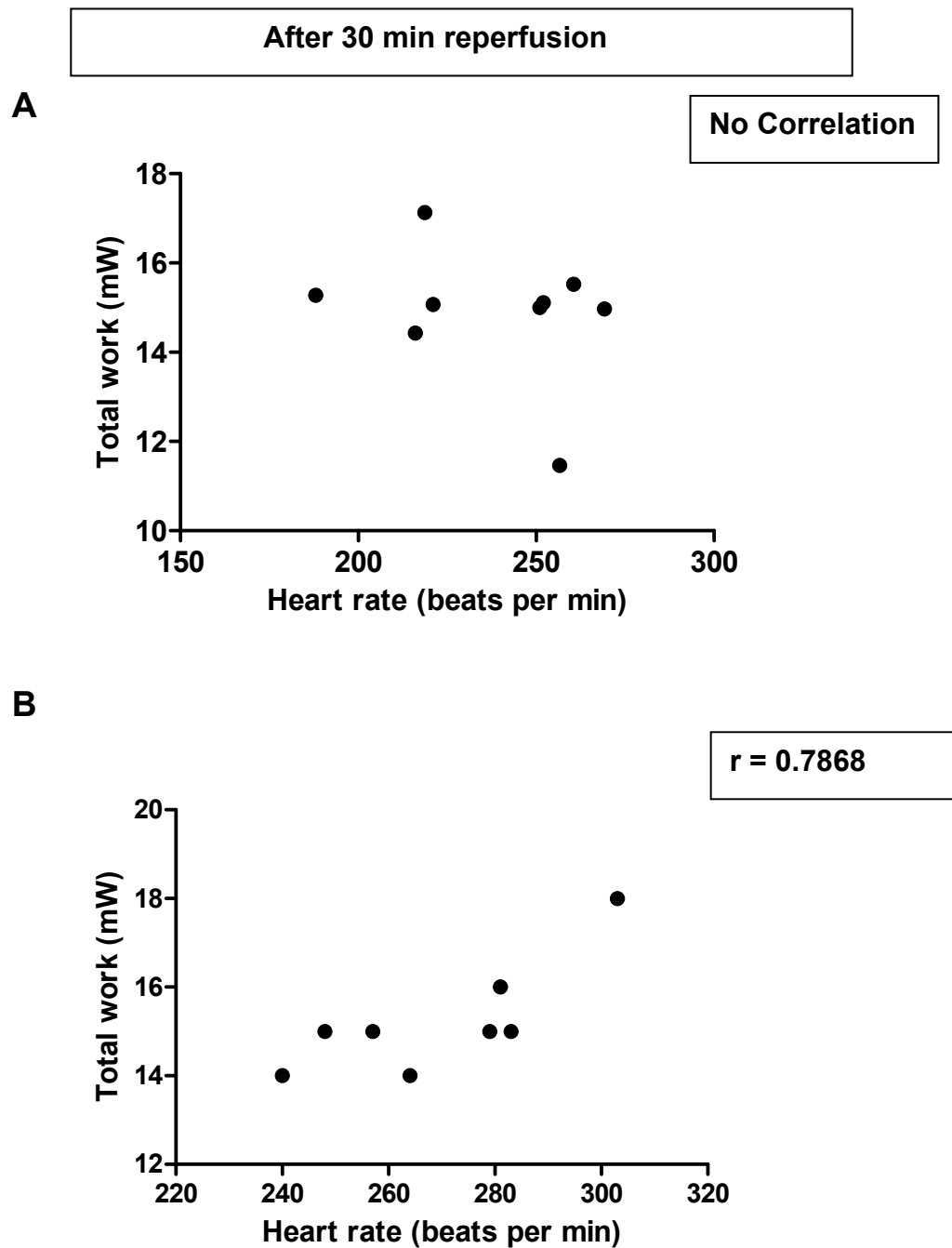


Figure 5.3.16 Heart rate and ejection fraction, measured at baseline/stabilisation (15 min working heart), between IPC hearts which showed functional recovery and failed respectively, after 30 min reperfusion (n=20).

**5.3.17 Comparison of the heart rate and total work obtained for the IPC group at baseline/stabilisation and functional recovery at 30 min reperfusion.**



**Figure 5.3.17 A & B** Correlation of heart rate with total work, measured at baseline/stabilisation (15 min working heart), between IPC hearts which showed functional recovery (indicated in **A**) and failed respectively (indicated in **B**), after 30 min reperfusion (n=8).

## 5.4) Discussion

Several studies demonstrated the involvement of various MAPK signaling cascades in the cardioprotective mechanism of IPC. The p38 MAPK pathway is the most controversial pathway studied in the context of IPC. The published data are inconsistent and therefore the role of p38 in IPC still remains unclear. However, it is generally believed that in the isolated working rat heart model, an attenuation of p38 activation is associated with IPC-induced functional recovery, after 30 min reperfusion [Marais et al., 2001]. Our results however, did not follow the expected pattern of p38 MAPK activation, but showed variable responses between different animals, as indicated in **Figure 5.3.1 A, B & C, Figure 5.3.2 A, B & C and Figure 5.3.3 A, B & C**. PKB/Akt also showed variable responses between different animals, but to a lesser extent compared to p38 MAPK.

It is also well established that the prosurvival kinases, PKB/AKT and ERK p44/p42 are associated with IPC induced protection. It has been shown that PKB/AKT and ERK p44/p42 are phosphorylated during early (15 min) reperfusion [Hausenloy & Yellon, 2004]. These two prosurvival kinases are denoted as “reperfusion injury salvage kinase” (RISK) pathways and are reported to protect the myocardium against ischaemia/reperfusion damage. Although Hausenloy & Yellon [2004] have reported a biphasic activation of PKB just after IPC and after 15 min reperfusion, it might be that at 30 min reperfusion PKB returned to baseline levels, which is in agreement with our results.

The next aspect that was investigated in the present study was the activation patterns of p38 MAPK and the RISK pathway in the IPC group, between hearts from animals that showed functional recovery after 30 min reperfusion and hearts that failed (**Fig. 5.3.4. A, B & C**).

Interestingly, hearts that showed 49% and 58% functional recovery, showed in some animals the same level of p38 MAPK activation as failed hearts (**Figure 5.3.4 A**), whereas some failed hearts showed lower levels of p38 MAPK activation as those that showed 49% and 58% recovery (**Figure 5.3.4 A**). Furthermore, PKB/AKT activation was more pronounced in failed hearts from some animals than hearts that showed functional recovery (**Figure 5.3.4 C**).

A further aspect that was investigated was the patterns of p38 MAPK activation and involvement of the RISK pathway in IPC hearts which failed after 30 min reperfusion (**Figure 5.3.5 A, B & C**). Hearts from this group of animals also showed a variable pattern of p38 MAPK activation (**Figure 5.3.5 A**). The RISK pathway **Figure 5.3.5 B & C** was also activated in this group, which is quite unexpected, since the IPC hearts showed no functional recovery after 30 min reperfusion and according to the literature activation of the RISK pathway is associated with cardioprotection [Hausenloy & Yellon, 2004].

Even more intriguing was the finding that the RISK pathway was also activated in the non-IPC hearts after 30 min reperfusion (**Figures 5.3.6 B & C**). In addition the small heat shock protein-27 (HSP-27), associated with cardioprotection, was also activated in the non-IPC group (**Figure 5.3.6 D**).

Our results indicated that in the majority of animals (85%), there were no significant differences in the activation of p38 MAPK between the IPC group and non-IPC group after 30 min reperfusion. This resulted in no significant differences in activation of the RISK pathway and HSP-27 between IPC and non-IPC (data not shown).

In some groups of animals (15%) however, significant differences were observed in p38 MAPK activation between the IPC group and non-IPC group after 30 min reperfusion (**Figure 5.3.8 A**). This was accompanied by significant differences in the activation of the RISK pathway and HSP-27 after 30 min reperfusion. p38 MAPK activation was increased in the non-IPC group (**Figure 5.3.8 A**), as expected. Interestingly, the RISK pathway (**Figure 5.3.8 B and C**) and HSP-27 (**Figure 5.3.8 D**) activation was also increased in the non-IPC group, compare to the IPC group.

Our data consistently show an attenuation of PKB/AKT activation at 15 min (**Chapter 3, Figure 3.3.1(b)C**) and 30 min reperfusion (**Chapter 3, Figure 3.3.1(b)D**) in the IPC groups, compared to the non-IPC groups. This reduced activation of PKB/Akt was always associated with a significant attenuation of p38 MAPK, ERKp44/p42 and HSP-27 in the IPC groups compared to the non-IPC groups. These results may suggest that an attenuation of p38 MAPK kinase



activation in the IPC group after reperfusion is detrimental to the ischaemic heart, since activation of the protective RISK pathway follow the same pattern as p38 MAPK activation. Alternatively, these results may suggest that activation of the RISK pathway in the IPC group after 30 min reperfusion is not associated with functional recovery of the ischaemic heart.

In order to investigate these aspects, correlation studies were performed with the IPC groups, to establish whether functional recovery in the IPC group correlated with an attenuation of p38 MAPK activation and subsequent activation of the RISK pathway. Results from the present study showed no correlation between ERKp44 and ERKp42 activation respectively, in the IPC group at 30 min reperfusion and functional recovery at 30 min reperfusion. Similarly, no correlation was observed between ERKp42 activation in the IPC group at 30 min reperfusion and functional recovery at 30 min reperfusion. Interestingly, no correlation was observed between activation of PKB/Akt in the IPC group at 30 min reperfusion and functional recovery at 30 min reperfusion. According to the literature, PKB/Akt and ERK MAPK denoted as the RISK pathway is associated with functional recovery in IPC during reperfusion [Hausenloy et al., 2005], in which case a positive correlation ( $r = +1$ ) is expected in the present study. No correlation however, was observed between functional recovery during IPC at 30 min reperfusion and activation of the RISK pathway at 30 min reperfusion. In addition, the results also failed to show any correlation between p38 MAPK activation at 30 min reperfusion and functional recovery in the IPC group at 30 min reperfusion. According to the literature functional recovery in the IPC group at 30 min reperfusion correlates with an attenuation of p38 MAPK activation [Marais et al., 2001], in which case one would expect a negative correlation ( $r = -1$ ) in the present study. No correlation however, was observed between p38 MAPK and functional recovery in IPC at 30 min reperfusion.

The second aim for this part of the study was to investigate the variable signaling patterns of kinase activation in the IPC group, observed at all time points of reperfusion and also in control hearts before and after sustained ischaemia. Since the variable patterns of kinase activation could not be attributed to differences in functional recovery the aim of this part of the study was to identify the parameters associated with the variable signaling responses.

The results demonstrated that the following haemodynamic parameters measured at baseline/stabilisation (15 min working heart) and thus before the start of the experiment, had a significant effect on kinase activation or attenuation: Aortic output (AO), Coronary output (CO), Heart Rate (HR), Aortic diastolic pressure, Peak systolic pressure (PSP), Ejection, Total work (Wt),  $dP/dT_{Min}$  and  $dT/dT_{Max}$ . The results from the present study demonstrated that these parameters effect kinase activation and attenuation in control hearts, after 25 min sustained ischaemia, after the IPC protocol (3xIPC), IPC after 5, 10 and 30 min reperfusion.

The results from this part of the study demonstrated that activation of the RISK pathway (PKB/Akt & ERK p42) and GSK-3 $\beta$  in the IPC group at 30 min reperfusion correlated positively with heart rate measured at baseline/stabilisation (15 min working heart). These results implicate that the activation of PKB/Akt, ERK p42 and GSK-3 $\beta$  correlate positively with nervous system (sympathetic) stimulation. This is a novel finding and not reported in the literature. This finding can explain why activation of PKB/Akt and ERK MAPK generally show a similar activation pattern during reperfusion. This can also explain why GSK-3 $\beta$  show a similar activation pattern as PKB/Akt during reperfusion. These results implicate that the higher heart rate measured at baseline/stabilisation increases the phosphorylation of PKB/Akt, ERK p42 and GSK-3 $\beta$  at the end of reperfusion. Increased activation of PKB/Akt is known to be anti-apoptotic and phosphorylation and subsequent inactivation of GSK-3 $\beta$  will close the mitochondrial permeability transition pore (mPTP). Since closure of the mPTP is associated with cardioprotection, these results could explain the cardioprotection (measured as functional recovery) seen in this group of IPC hearts. On the contrary, as demonstrated in Fig. 5.3.16 a significant increase in heart rate measured at baseline/stabilisation (15 min working heart) in the IPC group resulted in failing of the hearts after 30 in reperfusion. An increase in heart rate will enhance the metabolic demand on the heart during ischaemia. A higher metabolic demand during ischaemia has been associated with reduced functional recovery as a result of an increase in infarct size. This could explain why these hearts (indicated in Fig.5.3.16) failed after 30 min reperfusion. These results implicate that a moderate increase in heart rate measured at baseline/stabilisation accompanied by a moderate activation of PKB/Akt in the

IPC group is cardioprotective. On the contrary, a significant increase in heart rate at baseline/stabilisation accompanied by overstimulation of PKB/Akt could result in failure of the heart after 30 min reperfusion.

In addition, activation of ERK p42 after the IPC protocol (3xIPC), showed a negative correlation with aortic output (AO) and cardiac output (CO). This indicates that a decrease in the output (performance of the heart) as measured at baseline/stabilisation (15 min working heart) is associated with an increased phosphorylation of ERK p42 after the IPC protocol (3xIPC). These results indicate that the attenuation of ERK p42 activation after the IPC protocol (3xIPC) is a measure of how well the heart was performing at baseline/stabilisation. These results implicate that an increased activation of ERK p42 after 3xIPC is not due to the effect of IPC, but correlates with the performance of the heart at baseline/stabilisation (15 min working heart). Since activation of ERK p42 is affected by sympathetic stimulation (positive correlation with heart rate at baseline/stabilisation), an increase in heart rate could affect the ejection of the hearts at baseline/stabilisation (15 min working heart) negatively, resulting in decreased performance (aortic and cardiac output) which correlate with an increased phosphorylation of ERK p42. Interestingly, ERK p44 activation/attenuation showed no correlation with the haemodynamic parameters in the present study.

Phosphorylation of PKB/Akt in the IPC group after 5 min reperfusion correlated negatively with aortic diastolic pressure and correlated positively with the minimum rate of contraction ( $dP/dT_{Min}$ ). These results implicate that an increased phosphorylation of PKB/Akt correlates with a decrease in aortic diastolic pressure, which is the lowest pressure in the aorta before the ejection of blood from the left ventricle. These results implicate that an increase in phosphorylation of PKB/Akt will therefore promote (enhance) relaxation and adequate refilling of the left ventricle in order to eject blood into the aorta. These results suggest that a moderate activation of PKB/Akt is beneficial to the heart, as overstimulation of PKB/Akt will decrease the diastolic pressure in the aorta below normal levels. At 10 min reperfusion however, phosphorylation of PKB/Akt correlated positively with peak systolic pressure (PSP) at baseline/stabilisation (15 min working heart). The pressure in the aorta rises when the left ventricle

ejects blood into the aorta. The maximum aortic pressure following ejection is known as the systolic pressure. These results implicate that an increased phosphorylation of PKB/Akt correlates with an increase in systolic pressure in the aorta, which is associated with the ejection of blood into the aorta. Phosphorylation of PKB/Akt after the IPC protocol (3xIPC) correlated positively with aortic diastolic pressure and negatively with peak systolic pressure (PSP) measured at baseline/stabilisation (15 min working heart). These results implicate that a decrease in PKB/Akt after the IPC protocol (3xIPC) correlates with a decrease in aortic diastolic pressure. The decreased diastolic pressure in the aorta is associated with relaxation and filling of the left ventricle, just before ejection of blood into the aorta. These results implicate that moderate activation of PKB/Akt may facilitate adequate filling and ejection of blood from the left ventricle. On the contrary, overstimulation of PKB/Akt may be detrimental to the heart, as the increased activation of PKB/Akt will increase the aortic diastolic pressure, thereby preventing adequate refilling and ejection of blood from the left ventricle into the aorta. These results may explain the controversies regarding PKB/Akt activation during IPC and early reperfusion. These results are novel and not available in the literature.

Expression of HSP-70 during IPC at 10 min reperfusion, correlated positively with heart rate at baseline/stabilisation (15 min working heart). These results suggest that phosphorylation of these kinases and HSP-70 expression are influenced by, or associated with nervous system stimulation. HSP-70 is a member of the heat shock protein family and serves as a molecular chaperone. It plays a major role in protecting cells against different cellular stressors, such as heat, hypoxia, oxidative stress, ultraviolet irradiation, apoptosis and necrosis [Zhang et al., 2012]. Despite its protective functions, HSP-70 is used as a marker of pre-slaughter stress in animals [Bao et al., 2008; Cvorovic et al., 1998], as discussed in Chapter 4 (**section 4.8.8**) of this thesis. Interestingly, in the present study, HSP-70 expression correlated negatively with heart rate and minimal rate of contraction ( $dP/dT_{Min}$ ) at baseline/stabilisation (15 min working heart) in control hearts before IPC. This indicates that expression of HSP-70 is attenuated with increased heart rate and increased minimum rate of contraction ( $dP/dT_{Min}$ ) in control hearts before IPC. After the IPC protocol (3xIPC), its expression is altered in such a way that it correlates positively with heart rate. This may suggest that

overexpression of HSP-70 in the IPC group may be detrimental to the heart, as indicated in the present study (**Figure 5.3.16**) where an increased heart rate at baseline/stabilisation (15 min working heart) before IPC, is detrimental to the heart and results in failing of the IPC hearts. HSP-27 showed no correlation with haemodynamic parameters in the present study.

The results from this part of the study demonstrated that activation of p38 MAPK after 25 min ischaemia correlated positively with aortic output at baseline/stabilisation (15 min working heart). As p38 MAPK is activated by heat and ischaemia as well as during sustained ischaemia, great care was taken to keep the temperature constant at 36.5°C for 25 min during the period of sustained ischaemia. Thus in the absence of any variation in temperature the circumstances were ideal to measure p38 MAPK activation. There was no correlation between p38 MAPK activation and heart rate in the present study. Therefore these results can be interpreted that when the aortic output of the hearts increase in working heart mode, the activation of p38 MAPK also increases. This is also a novel finding and no data in literature exist to confirm or refute this finding. Once activated p38 MAPK phosphorylates its downstream substrate MAPKAP2 which in turn phosphorylates the small heat shock protein 27 (HSP-27), which is associated with cardioprotection. HSP-27 stabilizes the integrity of the cytoskeleton during ischaemia, thereby exerting a protective role. In addition, p38 MAPK is known to enhance glycolysis. Increased activation of p38 MAPK during 25 min sustained ischaemia, may provide energy via anaerobic glycolysis to sustain the ischaemic heart in the absence of oxygen.

In addition, results demonstrated that activation of CREB after 25 min ischaemia showed a positive correlation with the maximum rate of contraction ( $dP/dT_{Max}$ ) at baseline/stabilisation (15 min working heart). These results implicate that increased activation of CREB correlates with the increased rate of ventricular pressure rise in early systole. Increased activation of CREB is therefore an indication of increase in left ventricular pressure.

Furthermore, phosphorylation of AMPK in control hearts (before sustained ischaemia or IPC) correlated negatively with the maximum rate of contraction ( $dP/dT_{Max}$ ) at baseline/stabilisation (15 min working heart). In addition,

phosphorylation of AMPK after 3xIPC correlated negatively with peak systolic pressure and correlated positively with aortic diastolic pressure. These results implicate that phosphorylation of AMPK is associated with an increase in aortic diastolic pressure. Increased phosphorylation of AMPK will therefore decreased the relaxation and filling of the left ventricle, resulting in inadequate ejection of blood from the ventricle into the aorta. These results are also novel and not available in the literature.

Stenslokken and co-workers (2009) investigated the effect of harvesting, preparing and perfusing isolated mouse and rats hearts on kinase phosphorylation. They compared three different modes of perfusion: Langendorff-perfusion without an intraventricular balloon, Langendorff-perfusion with an intraventricular balloon and working heart mode. Their study demonstrated that phosphorylation of ERK p44/p42, JNK and p38 MAPK increased both by excising the heart and the mode of perfusion [Stenslokken et al., 2009]. Harvesting of the heart and mounting it onto the perfusion system were the least important determinants of kinase activation. The mode of perfusion however, plays an important role in kinase phosphorylation. Langendorff-perfusion with the intraventricular balloon induced the strongest effect on kinase phosphorylation in both rats and mice. Perfusion in working heart mode caused less phosphorylation than Langendorff perfusion with the balloon. They concluded that working heart modus may be a better model than Langendorff-perfusion for studies on IPC and postconditioning [Stenslokken et al., 2009]. No correlation studies regarding kinase activation and the haemodynamic parameters of the heart were conducted in their study [Stenslokken et al., 2009].

Results from this part of the study demonstrated that phosphorylation of CAMKII in the IPC group after 30 min reperfusion correlated negatively with aortic output (AO), cardiac output (CO) and total work (Wt) at 30 min reperfusion. These results indicate that an attenuation of CAMKII activation after 30 min reperfusion is associated with improved functional recovery, measured as AO and CO during IPC. This is also a novel finding and to our knowledge has never been reported before. According to the literature, CAMKII is activated in the myocardium by excessive catecholamine exposure, followed by chronic  $\beta$ -adrenergic stimulation [Anderson 2009]. Enhanced activation and expression of CAMKII is associated

with ischaemia/reperfusion injury, cardiac hypertrophy, myocardial cell death and heart failure [Mei-ling et al., 2012]. Attenuation of CAMKII activation protects against excessive  $\beta$ 1- adrenergic induced apoptosis [Zhu et al., 2003; Zhang et al., 2005; Yang et al., 2006]. Therefore the results from the present correlation study combined with what is known in the literature, suggest that hearts with decreased functional recovery after IPC at 30 min reperfusion and increased activation of CAMKII, were exposed to excessive catecholamine stimulation or chronic  $\beta$ -adrenergic activation.

To confirm this, we investigated the parameters between IPC hearts (used in this correlation study) that showed improved functional recovery during IPC after 30 min reperfusion and IPC hearts that did not recover after 30 min reperfusion. As indicated in **Figure 5.3.16 A & B** the results demonstrated that the only significant differences in the haemodynamic parameters between these two sets of hearts were that heart rate measured at 15 min working heart was significantly increased in the failed hearts compared to the hearts that recovered. Accordingly, the ejection fraction measured at 15 min working heart was significantly decreased in the failed hearts compared to the ejection fraction of the hearts that showed functional recovery. Additionally, the increased heart rate of the failed IPC hearts showed a positive correlation with the total work (Wt) performed at 15 min working heart (**Figure 5.3.17 B**). The IPC hearts that showed functional recovery at 30 min reperfusion however, showed no correlation with the total work (Wt) performed at 15 min working heart (**Figure 5.3.17 A**). These results suggest that an increased heart rate (as in failed hearts) influences the ejection of the heart negatively; therefore the heart has to increase its performance (total work) to be able to eject blood. In the hearts that showed functional recovery however, the ejection fraction was effective enough as a result of the decreased heart rate, to eject blood from the left ventricle without affecting the work load on the heart.

## 5.5) Conclusion

In this part of the present study our aims were to correlate activation of the RISK pathway, p38 MAPK and kinases investigated in Chapter 3 of the thesis with functional recovery during IPC at 30 min reperfusion. The second aim was to



investigate the variable signaling patterns of kinase activation during IPC and to identify the parameters involved or responsible for the variability in kinase activation observed during all time points investigated.

Results from the present study confirm that activation of the majority of the kinases investigated in Chapter 3, do not correlate with functional recovery during IPC after 30 min reperfusion, but correlate positively or negatively with the haemodynamic parameters of the heart, as measured after 15 min working heart. Since this time point is directly after the stabilisation phase before the start of the experiment (before IPC), it may suggest that IPC per se, does not induce or activate these kinases, which includes the RISK pathway, but that their activation patterns are induced by events before the experiment commences.

Activation of the majority of the kinases at different time points, correlate with heart rate before the start of the experiment as measured after 15 min working heart (before the IPC protocol, or sustained ischaemia). This indicates that activation of these kinases are influenced by nervous system stimulation before the experiment starts and while the animals are still alive.

In addition, the correlation results explain the variable signaling patterns of kinase activation observed at the different time points before and after sustained ischaemia, after the IPC protocol and also during the different reperfusion time points in the present study. The variable signaling patterns could be attributed to differences in heart rate and the effect thereof (ejection fraction, minimum and maximum rate of contraction), as a result of stress related processes in the animals before slaughtering. Since the animals utilized in the present study, were randomly assigned to different groups (IPC and non-IPC) before slaughtering and not exposed to any physical or emotional stress induced procedures such as maternal separation, foot shocks, transport or food and water deprivation, we hypothesize that increases in heart rate is due to emotional stress experienced by the animals in the laboratory as a result of pre-slaughter stress.

The results from the proteomic study discussed in Chapter 4 of the thesis demonstrated that the non-IPC hearts possess over the remarkable ability to activate protective processes to overcome the damaging effects of sustained



ischaemia. Therefore we suggest that non-IPC hearts are not the ideal negative controls for IPC hearts, in order to investigate the mechanism of IPC induced protection. Subsequently in this part of the study, we compared IPC hearts which showed functional recovery with IPC hearts that failed. Results demonstrated that IPC hearts failed as a result of increased heart rate leading to a decreased in ejection fraction. As a result of this, these hearts have to increase their performance (total work and  $dP/dT$  Max) to be able to eject blood from the left ventricle and may end up with low glucose levels before the start of sustained ischaemia. Since anaerobic glycolysis is the only source of ATP generation during ischaemia, an attenuation of glycolysis during ischaemia and reperfusion are detrimental to the ischaemic heart as fatty acid oxidation will dominate (discussed in Chapter 4 of the thesis). The results in this part of the study therefore support the results obtained in Chapter 4.

In addition, we identified CAMKII as a marker of functional recovery after IPC. Results from this part of the study demonstrated that an attenuation of CAMKII activation during IPC at 30 min reperfusion is associated with functional recovery of the ischaemic heart. According to our results attenuation of CAMKII activation is a more sensitive and reliable indicator or marker of functional recovery than p38 MAPK. This is a novel finding and to our knowledge has never been reported before.

Importantly, the results presented in this part of the study could also explain the controversies in the literature regarding activation/attenuation patterns of the different protein kinases at different time points of reperfusion, as discussed in the literature review (Chapter 1 of the thesis).

## Chapter 6

### Final conclusions and future studies

In the present study, one of the most important observations made was that activation of the majority of the protein kinases investigated, are associated with the haemodynamic parameters of the heart instead of functional recovery at the end of reperfusion. This is a novel finding and to our knowledge has never been reported before. Importantly, this has major implications for cell based research studies where kinase activation/attenuation is associated with IPC-induced cardioprotection in a specific context, without the necessary contribution of mechanical functional recovery measurements and haemodynamic parameters.

The results from the present study can explain the variable signaling patterns of kinase activation observed at the different time points before and after sustained ischaemia, after the IPC protocol and also during the different reperfusion time points investigated in the present study. Results indicated that the variable signaling patterns could be attributed to differences in heart rate and the effect thereof (ejection fraction, minimum and maximum rate of contraction), as a result of sympathetic stimulation due to psychological stress in the animals before slaughtering. These results suggest that IPC does not induce activation /attenuation of these kinases, including the RISK pathway, but their activation patterns is a result of sympathetic stimulation when the animal is still alive.

Importantly, these novel findings could help explain the controversies in the literature regarding activation/attenuation patterns of the different protein kinases during IPC, as discussed in the literature review (Chapter 1 of this manuscript). Therefore, data should be interpreted with great caution especially where activation /attenuation of the RISK pathway and p38 MAPK are investigated.

Proteomic data revealed an important role for proteins related and also unrelated to cardiac metabolism. The expression of the protective HSP's implicated in protein import and protein folding under stress conditions is increased in non-IPC and interestingly, these HSP's are also associated with pre-slaughter stress. Of

particular interest was the observation that the expression of Annexin A3, implicated in cell regeneration was upregulated in non-IPC compared to IPC. The upregulation of Annexin A3 may induce protective processes, leading to the increased expression of proteins related to cardiac metabolism, as observed in non-IPC.

These results suggest that non-IPC has an endogenous protective mechanism, which comes into play under various stress situations, whereby the induction of the protective HSP's and Annexin A3 are upregulated when oxygen and nutrients are re-introduced in the post-ischaemic early reperfusion phase. The non-IPC possess the remarkable ability to induce adaptive processes to reverse the damaging effects of sustained ischaemia and the adverse effects of enhanced sympathetic stimulation.

It is well documented that the diseased heart cannot be preconditioned [Cokkinos et al., 2003; Moolman et al., 1992]. Results from the present study however, suggest that the preconditioned heart is more susceptible to the adverse effects of chronic beta stimulation than a non-IPC heart, probably as a result of the repetitive short episodes of ischaemia during the IPC protocol. This may result in poor functional recovery or failure of the IPC hearts, as a result of the altered metabolism, demonstrated by the attenuation of glycolysis and the altered transport of fatty acids. This is a novel finding and to our knowledge has never been reported before. It is well established, however, that an increased heart rate increases the severity of the ischaemic injury [Shaper 1987] and chronic  $\beta$ -adrenergic receptor stimulation increases oxidative stress, leading to catecholamine-induced cardiotoxicity [Zhang et al., 2005; Remondino et al., 2003; Zhang et al., 2007].

Taken together, these results demonstrated that an IPC heart which failed after ischaemia /reperfusion is metabolically compromised and "worse off" compared to a non-IPC heart. To our knowledge this is the only study that investigated this aspect in the context of IPC during reperfusion. In addition, the results from this part of the study suggests that chronic beta stimulation (before a period of sustained ischaemia) can result in poor recovery of the ischaemic heart.

In addition, we identified CAMKII as a novel marker of functional recovery after IPC. The results demonstrated that an attenuation of CAMKII activation during IPC at 30 min reperfusion is associated with functional recovery of the ischaemic heart. According to our results attenuation of CAMKII activation is a more sensitive and reliable indicator or marker of functional recovery than p38 MAPK. This is a novel finding and to our knowledge has never been reported before.

## **Shortcomings of the present study**

When we set out to do this study, parts of our aims were:

- a) to do a Proteomic study on the membrane fraction of IPC and Non-IPC heart tissue to measure the density of beta receptor population.
- b) to confirm HSP-60, HSP-70, AMPK, CREB GSK-3 $\beta$  and CAMKII activation and expression during IPC at 15 min reperfusion with Western blots.

Unfortunately these tissue samples were accidentally destroyed by a colleague and lack of this data is definitely part of the shortcomings of the study.

Measurement of corticosterone, adrenalin, vasopressin and T-BARS levels in plasma and fatty acid determinations in tissue would have contributed to the novelty of the study. These measurements are not routinely done and since the proteomic study was an example of “data-mining” it was impossible to know beforehand which proteins are differentially regulated between IPC and non-IPC hearts.

## **Future studies**

These novel findings do not only explain the possible differences that exist at protein level between an IPC that failed or showed poor functional recovery at reperfusion and non-IPC, but demonstrated that non-IPC hearts possess over the remarkable ability to reverse the adverse effects of sympathetic stimulation as well as the damaging effects of sustained ischaemia. Therefore, this protective mechanism of non-IPC is an ideal or promising model for future investigations of cardioprotective strategies. It is highly recommended to measure corticosterone, adrenalin and vasopressin levels in the plasma of animals as indicators of

psychological stress before sacrificing and to correlate it with HSP-20 and HSP-70 expression levels as markers of pre-slaughter stress. It is also important to evaluate signaling events during early (5 min and 10 min) and late (30 min) reperfusion time points in combination with haemodynamic and functional recovery parameters to correlate with hearts that failed or showed functional recovery. Proteomic technologies can also contribute to these findings. This is essential for the clinical application of cardioprotective strategies in the future.

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